Is Anti-Müllerian Hormone a Hormone?

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Abstract

The aim of this thesis is to investigate if Anti-Müllerian hormone (AMH) is a hormone. AMH is a protein produced in the testes and ovaries. The classical function of AMH is to regulate Müllerian duct regression in the male fetus. However, AMH continues to be expressed in males after this time for unknown reasons. AMH is present at very high levels in the blood of males during infancy and childhood. After puberty, AMH levels are present at lower levels in men. Furthermore, AMH is also present in women from puberty until menopause.

It is not known why AMH is secreted into the bloodstream or why its levels are variable throughout development. Therefore, despite its name it is not known if AMH is a hormone. To earn this classification an endocrine function for AMH must be identified. That is, circulating AMH must be shown to interact with receptors in cells outside the gonads to induce an effect of some kind.

A preliminary screen of AMHRII expressing tissues was performed with an AMHRII reporter mouse. This analysis suggested that AMHRII expression is extensive in the fetus including cells of the cardiovascular, skeletal, respiratory, digestive, nervous and integumentary systems. While analysis in the adult AMHRII reporter mouse suggested AMHRII expression was found in the cardiovascular system and cartilage.

Findings from a correlative study comparing AMH levels and cardiovascular measures in healthy men indicated that AMH levels inversely correlate with aortic diameter independent of other known determinants for this measure. This suggests that AMH may be a regulator of blood vessel size. Consistent with this AMH levels were significantly altered in abdominal aortic aneurysms and varicose veins which are both conditions of pathological blood vessel enlargement.
Correlative studies in healthy school boys showed that AMH levels inversely correlated with height and the proportion of adult growth the boys had achieved. This indicates that AMH may be a hormonal regulator of bone maturation. However, Inhibin B also correlated with boy height measures which confounded interpretation of the results. Animal studies showed that adult AMH−/− mice of both sexes had significantly shorter bones than their wildtype littermates. This is consistent with AMH having a direct action on bone, although the growth and bone measurements were not altered in young AMH−/− mice which indicates this association is complex.

Further human studies, found that AMH levels correlate with vitamin D levels in men and women. Additionally, AMH levels and vitamin D levels were both shown to decrease in winter with vitamin D supplementation sufficient to prevent the winter decline in AMH levels. This indicates that AMH levels are regulated by vitamin D through a previously characterised vitamin D element in the AMH gene.

This thesis, presents the first evidence that AMH has putative hormonal functions in humans and that AMH concentration in the blood is regulated. This suggests that AMH found in the blood is not simply leakage from the gonads but that AMH is secreted to perform endocrine signalling.
Publications

The results from this thesis appear in the following publications. Other manuscripts are in preparation.


**Dennis, N.A.;** Jones, G.T.; van Rij, A.M.; McLennan, I.S. *Anti-Müllerian hormone may be a hormonal regulator of the cardiovascular system.* Endocrine Abstracts (2012) 29 P311

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# Table of Contents

**Abstract** ........................................................................................................................................................................... 2

**Publications** ........................................................................................................................................................................... 4

**Acknowledgements** .............................................................................................................................................................. 5

**Table of Contents** ..................................................................................................................................................................... 6

**List of Figures** .......................................................................................................................................................................... 12

**List of Tables** ........................................................................................................................................................................... 14

**List of Abbreviations** .............................................................................................................................................................. 15

**Chapter 1: General Introduction** ............................................................................................................................................... 16

1.1 Structure of thesis .................................................................................................................................................................. 16

1.2 Characteristics of a hormone .................................................................................................................................................. 16

1.3 Anti-Müllerian hormone – the basics .................................................................................................................................. 17

1.4 Circulating AMH ..................................................................................................................................................................... 18

1.5 AMH receptors and signalling ................................................................................................................................................. 21

1.6 Known functions of AMH signalling ................................................................................................................................... 25

   1.6.1 Development of the reproductive organs ...................................................................................................................... 25

   1.6.2 Gonadal development and steroidogenesis ................................................................................................................... 26

   1.6.3 Development of the nervous system ............................................................................................................................. 27
1.6.4 **Lung development** ................................................................. 28

1.6.5 **Fertility** ....................................................................................... 28

1.6.5.1 Follicle regulation ........................................................................ 28

1.6.5.2 Sperm Viability ............................................................................. 30

1.7 **AMH and cell growth regulation** ................................................... 30

1.8 **Is AMH a hormone?** ...................................................................... 32

**Chapter 2: Location of the AMHRII Receptor** .................................... 34

2.1 **AMHRII reporter mouse** ................................................................. 34

2.2 **Results** ........................................................................................... 37

2.2.1 **Fetal AMHRII reporter mouse** .................................................. 37

2.2.2 **Fetal eye** ..................................................................................... 37

2.2.3 **Fetal epithelium** .......................................................................... 41

2.2.4 **Fetal muscle** ................................................................................. 43

2.2.5 **Fetal bone** ................................................................................... 45

2.2.6 **Fetal visceral organs** ................................................................... 45

2.2.7 **Adult AMHRII reporter mouse** .................................................. 47

2.3 **Discussion** ....................................................................................... 51

2.4 **Recommendations for further research** .......................................... 55

**Chapter 3: AMH and Blood Vessels** ................................................. 57

3.1 **Introduction** .................................................................................... 57

3.1.1 **Physiology of blood vessels** ...................................................... 57
3.1.2 Vascular remodelling .......................................................... 58

3.1.3 Blood vessel dilation .......................................................... 60

3.1.4 TGF superfamily and blood vessels ..................................... 61

3.2 Results ................................................................................. 63

3.2.1 AMH negatively correlates with Abdominal Aortic diameter .......... 63

3.2.2 Aortic diameter did not correlate with other testicular hormones ........ 68

3.2.3 AMH correlated with aortic diameter independent of body size .......... 69

3.2.4 AMH correlated with aortic diameter independent of cardiovascular risk factors .... 72

3.2.5 Abdominal aortic aneurism patients had lower serum AMH ............. 76

3.2.6 AMH levels in other blood vessel diseases .................................. 77

3.3 Discussion ............................................................................ 81

3.4 Recommendations for further research ..................................... 85

Chapter 4: AMH and Growth ....................................................... 87

4.1 Introduction ........................................................................ 87

4.1.1 Postnatal growth ............................................................. 87

4.1.2 Human growth ............................................................... 88

4.1.3 Bone growth ................................................................. 89

4.1.4 Endocrine regulators of the growth plate ............................... 90

4.1.5 Regulation of pubertal onset ............................................. 92

4.2 Results: Part (A) AMH and child growth ................................. 94

4.2.1 AMH was sexually dimorphic and highly variable in children ........ 94
4.2.2 AMH levels correlated with height and finger length in boys .......................... 94
4.2.3 AMH levels correlated with growth maturity in boys ........................................ 100
4.2.4 The correlation between AMH and growth is independent of other growth hormones. 101
4.2.5 Inhibin B also correlated with growth measures............................................. 104

4.3 Part (B) AMH and mouse growth ........................................................................ 106
4.3.1 Bone length was not significantly reduced in young AMH−/− mice .................... 106
4.3.2 Early growth was not affected in AMH deficient mice ....................................... 109
4.3.3 Adult bone length was reduced in AMH−/− mice .............................................. 114
4.3.4 AMH does not affect bone density ................................................................. 115

4.4 Discussion ........................................................................................................ 119
4.4.1 AMH may retard the development of boys ...................................................... 119
4.4.2 AMH and InhB may have redundant actions on growth .................................... 120
4.4.3 Mouse growth .............................................................................................. 122
4.4.4 Are the actions of AMH the same in mice and humans? ................................. 124

4.5 Recommendations for further research ............................................................ 126

Chapter 5: Regulation of AMH Expression ............................................................ 129

5.1 Introduction ...................................................................................................... 129
5.1.1 Transcriptional regulation of AMH expression ............................................... 130
5.1.2 Vitamin D response element ........................................................................ 131
5.1.3 Vitamin D .................................................................................................... 132

5.2 Results ............................................................................................................. 133
5.2.1 AMH levels correlated with vitamin D status in men .......................... 133
5.2.2 Vitamin D supplementation prevented the winter related decline of AMH in women --- 136
5.2.3 AMH did not correlate with vitamin D status in boys .......................... 137
5.2.4 The vitamin D response element is not conserved across species .................. 140
5.2.5 Other conserved areas in the AMH gene promoter ............................. 144

5.3 Discussion ............................................................................................. 144

5.4 Recommendations of further research .................................................. 148

Chapter 6: Final Conclusions ....................................................................... 150

6.1 AMHRII appears to be expressed extensively throughout the body .................... 150

6.2 Circulating AMH has putative functions ................................................. 151

6.3 Circulating AMH is biologically regulated .............................................. 152

6.4 AMH is a putative hormone ..................................................................... 153

6.5 Implications of AMH deficiency ............................................................... 154

6.6 Limitations of research .......................................................................... 157

Chapter 7: Methods ..................................................................................... 158

7.1 Human Studies ...................................................................................... 158

7.1.1 Men cohort ...................................................................................... 158

7.1.2 Child cohort .................................................................................... 159

7.1.3 Women cohort .................................................................................. 160

7.1.4 Serum Analysis ................................................................................. 161
7.1.5 Calculations

7.2 Mouse studies
7.2.1 Mice
7.2.2 LacZ reporter mouse histology
7.2.3 Growth study
7.2.4 Bone measurements mice
7.2.4.1 Vertebra
7.2.4.2 Tibia
7.2.4.3 Metatarsals
7.2.5 Bone Density
7.2.6 Vitamin D dosing study

7.3 AMH promoter sequence alignment

7.4 Statistical analysis

Chapter 8: References
List of Figures

Figure 1.1. AMH levels in human males and females..............................................................20
Figure 1.2. AMH levels do not vary throughout the ovarian cycle in women..............................20
Figure 1.3. TGF-B superfamily signalling..............................................................................23
Figure 4.25. Schematic of AMHRII reporter system...............................................................23
Figure 2.5. LacZ staining in a cross-section of the abdomen of E18 mouse fetuses................46
Figure 2.6. LacZ staining in a cross-section of the thorax of E18 mouse fetuses.......................39
Figure 2.7. LacZ staining in coronal sections of the eye in E18 mouse fetuses.........................40
Figure 2.8. LacZ staining in skin of E18 mouse fetus...............................................................40
Figure 2.9. Cross-section of intestine in E18 mouse fetuses......................................................42
Figure 2.10. LacZ staining in a coronal section of the nasal cavity of E18 mouse fetuses...........42
Figure 2.11. LacZ staining in fetal heart ...................................................................................44
Figure 2.12. LacZ staining in cross-section of a foot in E18 mouse fetuses...............................46
Figure 2.13. LacZ staining in the growth plate of E18 mouse embryos......................................46
Figure 2.14. LacZ staining in the adult AMHRII reporter mouse.............................................48
Figure 2.15. LacZ staining in the aortic wall of adult mice.......................................................49
Figure 3.16. Serum AMH levels did not correlate with age in mature men...............................65
Figure 3.17. Distribution of AMH levels in healthy men and men with abdominal aortic
aneurisms. ...............................................................................................................................65
Figure 3.18. AMH levels correlated with abdominal aortic diameter in healthy men.................66
Figure 3.19. AMH levels correlated with the ratio of the max-infrarenal and suprarenal aortic
diameters in healthy men......................................................................................................67
Figure 3.20. AMH levels correlate with InhB levels in healthy mature men...............................70
Figure 3.21. Distribution of AMH levels in men with varicose veins and men with peripheral
artery disease .............................................................................................................................79
Figure 3.22. Cumulative frequency of AMH levels in health men and men with vascular disease.
..................................................................................................................................................80
Figure 4.23. Serum levels of AMH and other gonadal hormones in human and mice males
during postnatal growth.........................................................................................................93
Figure 4.24. AMH levels compared with age in five- and six-year-old children........................96
Figure 4.25. AMH levels decrease with age in boys, but remain stable over one year..............96
Figure 4.26. AMH correlated with height and weight in boys..................................................97
Figure 4.27. AMH levels correlated with finger length in boys.................................................99
Figure 4.28. The levels of AMH, InhB and IGF-1 in five- and six-year-old boys......................103
Figure 4.29. Height correlated with serum InhB in five- and six-year-old boys.......................105
Figure 4.30. Measurements of the tibia in mice.......................................................................107
Figure 4.31. Tibia measurements are not significantly different in young AMH+/− mice........107
Figure 4.32. Vertebra measurements are not significantly different in young AMH+/− mice.....108
Figure 4.33. Weight of mice from AMH+/− x AMH+/− matings during early development......111
Figure 4.34. Daily growth rate in mouse pups was not affected by their AMH genotype........112
Figure 4.35. Tibia length was significantly shorter in adult AMH+/− mice...............................116
Figure 4.36. Adult AMH+/− male mice had significantly shorter caudal vertebra....................117
Figure 4.37. AMH had no significant effect on bone density....................................................118
Figure 5.38. AMH levels correlated with calcidiol concentration in men.................................135
Figure 5.39. AMH and calcidiol levels were seasonal................................................................. 138
Figure 5.40. Partial plot of linear regression presented in Table 5.16.......................................... 139
Figure 5.41. The levels of AMH did not correlate with calcidiol levels in boys......................... 139
Figure 5.42. Conserved regions in AMH gene promoter............................................................. 142
Figure 5.43. Alignment of the VDRE element in the AMH promoter in primates....................... 143
Figure 5.44. Vitamin D may not regulate AMH expression in mice........................................... 143
Figure 7.45 Measuring view of the fourth caudal vertebra in OsiriX image viewer...................... 169
Figure 7.46 Measuring view of the tibia in OsiriX image viewer................................................ 169
Figure 7.47 Measuring view of the second metatarsal in OsiriX image viewer............................ 170
List of Tables

Table 1.1. TGF-β superfamily type 1 receptors and example of their known roles ......................24
Table 3.2. Summary of LacZ staining results for fetal and adult AMHRII reporter mice ..........50
Table 3.3. Correlations between hormones and max-infrarenal aortic diameter .....................70
Table 3.4. Body size parameters correlated with max-infrarenal aortic diameter ......................71
Table 3.5. Body size parameters correlated with AMH concentration .....................................71
Table 3.6. AMH and aortic diameter correlations with atherosclerosis risk factors .................74
Table 3.7. Linear regression models for the prediction of maximum infrarenal aortic diameter .................................................................................................................................75
Table 3.8. Participant characteristics for healthy control and abdominal aortic aneurysm groups ..........................................................................................................................78
Table 3.9. Participant characteristics for varicose vein and peripheral artery disease groups 78
Table 4.10. Models predicting height in five- and six-year-old boys ......................................98
Table 4.11. Determinants of AMH, InhB and Height ...............................................................102
Table 4.12. There was no significant difference in metatarsal length in young AMH+/− male mice ..........................................................................................................................108
Table 4.13. Weight of AMH+/− and AMH−/− mice compared to AMH+/+ mice at development milestones ..................................................................................................................113
Table 4.14. Metatarsal length of 140-day-old mice .................................................................117
Table 5.15. Calcidiol covaries with AMH independent of InhB and age in 113 mature men 135
Table 5.16. Changes in AMH levels correlated with seasonal changes in calcidiol levels in 33 women ................................................................................................................................138
Table 7.17. Commercial ELISA kits used in this thesis .........................................................162
Table 7.18. Characteristics of mice used in bone scan study ...............................................166
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>Abdominal aortic aneurysm</td>
</tr>
<tr>
<td>ABI</td>
<td>Ankle brachial (pressure) index</td>
</tr>
<tr>
<td>Act</td>
<td>Activin</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-Müllerian hormone</td>
</tr>
<tr>
<td>AMHRII</td>
<td>Anti-Müllerian hormone receptor II</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAX-1</td>
<td>Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E18</td>
<td>Embryonic day 18</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth differentiation factor</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin like growth factor 1</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Insulin like growth factor binding protein 3</td>
</tr>
<tr>
<td>InhB</td>
<td>Inhibin B</td>
</tr>
<tr>
<td>KS test</td>
<td>Kolmogorov-Smirnov test</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NRSF</td>
<td>Neuron restrictive silencing factor</td>
</tr>
<tr>
<td>PCOS</td>
<td>Poly cystic ovary syndrome</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMDS</td>
<td>Persistent Müllerian duct syndrome</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
</tr>
<tr>
<td>SF-1</td>
<td>Steroidogenic factor 1</td>
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<tr>
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<td>SRY</td>
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<td>T3</td>
<td>Triiodothyronine</td>
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<tr>
<td>T4</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>t-test</td>
<td>Student’s t-test</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D responsive element</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilms tumour protein 1</td>
</tr>
<tr>
<td>Xgal</td>
<td>5-bromo-4-chloro-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>µCT</td>
<td>micro computed tomography</td>
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Chapter 1: General Introduction

1.1 Structure of thesis

This thesis investigates whether Anti-Müllerian Hormone (AMH) is a hormone. This chapter (Chapter 1) discusses what criteria define a hormone and whether AMH meets these criteria based on the current literature. In the subsequent research chapters, this thesis aims to further define the hormonal functions of AMH. Chapter 2 is a preliminary survey of the sites of expression of the AMH receptor, AMHRII. Chapter 3 presents evidence that AMH may be a hormonal regulator of blood vessels. Chapter 4 examines potential hormonal roles of AMH in bone growth and the maturation of boys, whereas Chapter 5 investigates regulators of AMH expression. Final conclusions are drawn in Chapter 6 and the methods used in this thesis are documented in Chapter 7.

1.2 Characteristics of a hormone

Cells must communicate with each other to regulate development and maintain homeostasis within the body. Typically, a cell receives information in the form of chemicals that have been released by other cells. Information may come from cells close to the target cell (paracrine signalling) or from other parts of the body (endocrine signalling). Endocrine signalling uses the bloodstream to deliver chemical messages, collectively called hormones, to their target cells.

There are five characteristics that define hormones in general; 1) hormones are biosynthesised in the secreting cell, 2) hormones are secreted into the blood, 3) hormones are recognised by receptors in the target cell, 4) hormones cause a response in the target cell and 5) hormone concentrations in the blood change to convey information about changes within the body and its environment [3].
Hormones can be in the form of peptides or chemicals such as steroids. Steroid hormones such as testosterone and estradiol can diffuse into the target cell where they act on cytoplasmic receptors. Peptide hormones such as growth hormone and insulin interact with membrane bound receptors on the target cell.

### 1.3 Anti-Müllerian hormone – the basics

AMH (also known as Müllerian Inhibiting substance, MIS) is a protein found in the blood which is produced by the Sertoli cells of the testes or the granulosa cells of the ovaries [4, 5]. While the sites of AMH expression have not been extensively studied, some endogenous expression has been found in the brain [6] and prostate [7]. The AMH in the brain and prostate is either produced in small amounts or not secreted into the blood as removing the gonads in males or females reduces circulating AMH to below detectable levels indicating that the testes and ovaries are the only significant source of circulating AMH [8-10].

AMH is a member of the transforming growth factor-beta (TGF-β) superfamily. Like other members of this family AMH is expressed as a pro-protein that is activated by cleavage at a specific site. AMH is expressed as a 140kDa glycoprotein precursor made of two 70kDa homodimers [11]. Pro-AMH is then proteolytically cleaved to produce an active C-terminal peptide (25kDa) and a larger (57kDa) N-terminal peptide which is thought to be inactive [12, 13]. It is not known where this cleavage takes place (i.e. in the gonads, blood or other tissue) or if the N- and C-terminal units remain associated in the blood. As per convention, AMH concentrations in this thesis assume a molecular weight of 140kDa for quantitating AMH in the blood.
1.4 Circulating AMH

Circulating AMH levels in the blood are influenced by sex and age. Human male embryos begin to express AMH in their testes at week 7 of gestation [14]. AMH expression continues throughout gestation in the male fetus and after birth. During infancy and childhood in boys, AMH is present in the blood at very high concentrations with AMH levels of 6 month old boys being around 20 times that of adult men. At present, it is not known why boys have higher AMH levels than adults. AMH is undetectable in the blood of young girls until close to puberty (Figure 1.1). Thus, during childhood circulating AMH is sexually dimorphic as it is present in boys but not girls. This sexual dimorphism in AMH production suggests that AMH signalling may regulate processes that are required for young males but not young females.

During puberty, AMH decreases in boys and increases in girls so that AMH levels are comparable in men and premenopausal women (Figure 1.1) indicating that AMH may have similar functions in young men and women. AMH levels have been shown to be stable over the course of the ovarian cycle in women despite considerable variation in other ovarian hormones such as estradiol, luteinizing hormone, follicle-stimulating hormone and the inhibins during the different phases of the ovarian cycle (Figure 1.2). This stability is possibly attributed to the long half-life of AMH in the blood which is nearly 28 hours in humans and an estimated 2 days in other species [15, 16]. The high stability of circulating AMH suggests that it may regulate processes that take a relatively long time.
Men continue to express AMH at much the same concentration throughout adulthood [17]. Conversely, as women age, their AMH levels progressively decline, until menopause when AMH ceases to be produced by the ovaries (Figure 1.1). As circulating AMH has not been ascribed a function in adults, the consequences of losing circulating AMH in menopausal women is not known. AMH exhibits a high inter-person variability in people of the same age and sex [17-19]. Little is known about why this variation exists or if people with low or high AMH levels have altered physiology.
Figure 1.1. AMH levels in human males and females.
AMH levels in the blood are very high during infancy and childhood in males (blue) and decline at puberty. Conversely, in females (purple), circulating AMH is undetectable during infancy and early childhood. AMH levels then increase at female puberty and reach peak levels in women when they are around 25 years old before they slowly decline to undetectable levels at menopause. Created with data from [17-19]. Note the y-axis has been split.

Figure 1.2. AMH levels do not vary throughout the ovarian cycle in women.
Estradiol, inhibin B, inhibin A, luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels all change throughout the different phases of the ovarian cycle in women. However, AMH levels remain constant. Created using data from [11, 15, 18, 20-23]
1.5 AMH receptors and signalling

AMH is a member of the transforming growth factor-beta (TGF-β) superfamily, a group of protein hormones and growth factors that use a distinctive form of signalling. In TGF-β signalling, the ligand (i.e. AMH) binds to a type II receptor (i.e. AMHRII) and to one of a variety of type I receptors (i.e. ALK2, ALK3 or ALK6). The type II receptor contains an intracellular kinase domain which phosphorylates the intracellular domain of the type I receptor. The activated type I receptor then recruits Smad proteins which translocate to the nucleus to regulate expression of their target genes (Figure 1.3).

Members of the TGF-β superfamily share the use of the type I (and occasionally the type 2) receptors. Just 7 type I receptors (ALK1-7) service over 30 ligands from the bone morphogenic protein (BMP), TGF-β, growth differentiation factor (GDF) and activin (Act) subfamilies (Table 1.1). By using different receptors, ligands can regulate a wide range of effects. For example, BMP7, which can use type II receptors BMPR-II, Act-IIA and Act-IIB with type I receptors ALK2, ALK3 or ALK6, can act as a neuronal survival factor [20], inhibit vascular calcification [21], promote growth and activation of brown adipose tissue [22] and act as an essential morphogen in renal, skeletal and eye development [23].

Like BMP-7, AMH also uses ALK2, ALK3 and ALK6 (Table 1.1) [24-26]. This suggests that AMH is capable of diverse roles that may be similar to ligands such as the BMPs and activins. BMP7 and AMH are already known to have similar roles as neuronal survival factors [6, 20] and this thesis will investigate the possibility that AMH may function as a hormonal regulator of blood vessels and bone, like BMP7 (Chapters 3 and 4). However, while the actions of the type I receptors give clues about the possible functions of AMH, it is difficult to gain information about AMH signalling alone from studying the type I receptors because of the complex interactions of the type I receptors with other TGF-β superfamily members (Figure 1.3).
AMH is, however, the only known ligand for the type II receptor, AMHRII. Studying AMHRII or more specifically the location of AMHRII can identify target tissues of AMH. AMHRII has been shown to be expressed in murine neurons [27] and fetal lungs [28], and human breast [29], endometrium [30] and prostate [31] in vivo as well as cervical cancer cell lines [32]. This indicates that AMH signalling may occur in a variety of tissues throughout the body although very few tissues have been studied. This thesis will undertake a provisional investigation of the location of the AMHRII in order to identify putative target cells for circulating AMH (Chapter 2).
Figure 1.3. TGF-B superfamily signalling.
Summary of interactions between members of the TGF-β superfamily members. Extracellular ligands (ovals) recruit both a type II receptor (green rectangles) and a type I receptor (blue rectangles) in the membrane. Receptors activate either the smad2/3 or smad1/5/8 pathway which acts on target genes. Ligands in red are present in blood and ligands that are not secreted into blood are coloured orange are not present in blood. AMH is drawn in purple for emphasis. Red broken lines indicate an inhibitory effect. Pathways have been simplified for clarity; inhibin and activin ligand subclasses have been grouped, as have the ActRII receptors; receptor modifiers betaglycan and endoglin have been omitted as have the GDF family of ligands. Created using data from [33-37]
### Table 1.1. TGF-β superfamily type 1 receptors and examples of their known roles

<table>
<thead>
<tr>
<th>Type I receptor</th>
<th>Alternative name</th>
<th>Ligands</th>
<th>Examples of regulatory roles</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK1</td>
<td>TSR1</td>
<td>TGF-β1,2,3 Activins</td>
<td>Proliferation and differentiation of endothelial cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMP-9,10</td>
<td>Activation of angiogenesis</td>
</tr>
<tr>
<td>ALK2</td>
<td>SKR1, ActRIA, ACVR1</td>
<td>AMH Activins</td>
<td>Müllerian duct regression</td>
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<tr>
<td></td>
<td></td>
<td>BMP-6,9</td>
<td>Heart morphology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGF-βs</td>
<td>Promotes chondrocyte differentiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Embryonic patterning</td>
</tr>
<tr>
<td>ALK3</td>
<td>BMPRIA, BRK1</td>
<td>AMH BMP-2,4,5,6,7,10</td>
<td>Müllerian duct regression</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Embryonic patterning</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GDF-5,6,9</td>
<td>Promotes osteoblast cell differentiation</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Heart morphology</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibits chondrocyte differentiation</td>
</tr>
<tr>
<td>ALK4</td>
<td>ActRIB, SKR2,ACVR1B</td>
<td>Activins</td>
<td>Embryonic patterning</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nodal</td>
<td>Heart morphology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myostatin</td>
<td>Inhibits pituitary cell proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inflammatory response</td>
</tr>
<tr>
<td>ALK5</td>
<td>TβRI</td>
<td>TGF-βs</td>
<td>Regulates extracellular matrix</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GDF9</td>
<td>Thyroid function</td>
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<tr>
<td></td>
<td></td>
<td>Myostatin</td>
<td>Cellular apoptosis</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Cellular migration and invasion</td>
</tr>
<tr>
<td>ALK6</td>
<td>BMPR1B</td>
<td>AMH BMP-2,4,5,6,7,10,15</td>
<td>Folliculogenesis and ovulation</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Appendicular Skeletal development</td>
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<tr>
<td></td>
<td></td>
<td>GDF-5,6,9</td>
<td>Differentiation of osteoblasts</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Postnatal bone formation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inhibits apoptosis of osteoblasts</td>
</tr>
<tr>
<td>ALK7</td>
<td>ACVR1C</td>
<td>Nodal</td>
<td>Embryonic patterning</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activins</td>
<td>Decreases cell proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increases cell apoptosis</td>
</tr>
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</table>

Type 1 receptors used by AMH in bold. Regulatory roles given are not an exhaustive list, but examples relevant to this thesis. Created from reviews [34, 37]
1.6 Known functions of AMH signalling

1.6.1 Development of the reproductive organs

AMH is named for its role in the regression of the Müllerian (paramesonephric) ducts in the male fetus [38]. All embryos begin life in a sexually ambiguous state containing both the precursors for the male sex organs, the Wolffian (mesonephric) ducts and the female sex organs, the Müllerian ducts [5]. If left intact, the Müllerian ducts develop into the uterus, uterine tubes, cervix and upper vagina [39]. In the male embryo, this is prevented by the expression of AMH in the testes (week 7 in humans) which mediates the regression of the cells of the Müllerian ducts [40].

Males lacking functional AMH, or its receptor AMHRII, are born with a rudimentary uterus attached to their testes, a condition termed persistent Müllerian duct syndrome (PMDS) [41]. Conversely, exposing a female fetus to AMH produces an apparently normal female lacking a uterus, uterine tubes and the upper third of the vagina [42]. Müllerian duct regression can only occur within the “critical period” of development, after which the Müllerian ducts do not regress if exposed to AMH [43]. This means that postnatal AMH expression does not cause regression in tissues derived from the Müllerian duct in females.

Originally, Müllerian duct regression was thought to be a hormonal function of AMH, with the characterisation of the freemartin cow. Freemartinism in cows occurs in twin pregnancies when the placentas of a male embryo and female embryo anastomose and the embryos share a blood supply. The AMH produced in the male twin causes the Müllerian ducts of the female twin to regress [44]. This is an example of ectopic AMH in the blood acting hormonally on the Müllerian ducts. However, unilateral Müllerian duct derivatives have been reported in human disorders where only one testis is present, such as mixed gonadal dysgenesis [45, 46], raising the possibility that Müllerian duct regression may occur through paracrine AMH signalling. In normal Müllerian duct regression, the tissue nearest to the testes regresses before tissue further
away from the source of AMH which is also consistent with a paracrine effect [40]. However, in species such as the chicken, where unilateral Müllerian duct regression is part of normal female development, Müllerian duct regression appears to be controlled by hormonal AMH with laterality mediated by the differential expression of the AMHRII receptor in the left and right Müllerian ducts [47].

Curiously, directed mutagenesis studies have shown that a three-fold decrease in AMH concentration does not induce PMDS in mice indicating that AMH is expressed in excess of what is required for Müllerian duct regression [3].

1.6.2 Gonadal development and steroidogenesis

The Leydig and Sertoli cells of the testes and the granulosa cells of the ovaries express AMHRII indicating that AMH may have paracrine actions within the gonads [48-50].

High doses of AMH decrease the expression of steroidogenic enzymes such as aromatase and the production of testosterone in cultures of Sertoli and Leydig cells [48, 51-56]. These enzymes facilitate the synthesis of steroid hormones such as testosterone and estrogen. AMH also decreases the production of the luteinizing hormone (LH) receptor in the testes [48, 52, 53, 57]. LH is secreted from the brain and increases androgen (such as testosterone) production in the testes. By blocking this signalling, AMH may further decrease the production of androgens in the testes.

Therefore, male mice over expressing AMH in their testes have symptoms of low androgen production such as impaired Wolffian duct development and have some female characteristics such as vaginal openings and nipples (note, unlike humans and other mammals, male mice do not have nipples)[42].
The Leydig cells are the main source of circulating testosterone in males. In addition to regulating Leydig activity, AMH also influences Leydig cell differentiation in the developing testis [48, 49]. AMH and AMHRII deficient mice have an increased number of Leydig cells in their testes [58, 59]. Conversely, mice over-expressing AMH have a decreased number of Leydig cells [48]. Curiously, AMH deficient mice have normal testosterone levels indicating that other systems (for example, feedback systems) may be in place to inhibit excess testosterone production or that the effect of AMH on testosterone synthesis has been overestimated.

1.6.3 Development of the nervous system

Most immature neurons in the murine brain and spinal cord express AMHRII [27, 60]. This raises the possibility that the fetal brain is reacting to AMH secreted from the fetal testes. Incubation with physiological levels of AMH improves the survival of mouse embryonic motor neurons and cortical neurons in culture suggesting that AMH mediates neuron survival [6, 60]. Consistent with this, AMH deficient mice have lower numbers of motor neurons in the spinal cord and a lower number of Purkinje neurons in the cerebellum of the brain [27, 61]. The number of these neurons is sexually dimorphic (i.e. different between males and females) with males having a greater number of motor neurons and Purkinje cells [62, 63]. In AMH deficient mice, the number of these neurons is decreased to female-like levels suggesting that AMH may contribute to the subtle sexual dimorphisms in the brain. Sexually dimorphic motor neuron traits such as the number of motor neurons in the bulbocavernosus nucleus are not affected by AMH indicating that AMH is not a general regulator of motor neuron survival [6].

AMH does not appear to be expressed in the embryonic brain [6] although in vitro studies suggest that embryonic neurons are capable of expressing AMH in culture [60]. Therefore, it is possible that the embryonic brain is a hormonal target of AMH signalling which may occur before the blood brain barrier forms [64].
It is not known if AMH can cross the blood brain barrier, but it is likely that AMH signalling in the mature brain occurs via paracrine and autocrine mechanisms because both AMH and AMHRII are expressed in mature neurons [6, 60, 65]. Like embryonic embryos, AMH is a neuronal survival factor in mature neurons [60].

Also, AMH deficient and AMHRII deficient mice behave similar to female mice in tests that analyse male-biased behaviour such as exploring new environments or objects [27, 66]. This indicates that AMH may be mediating some of the sexually dimorphic behavioural traits in the developing or adult brain.

1.6.4 Lung development

AMHRII is expressed in fetal lung tissue in mice [28] and high concentrations of AMH have been shown to decrease the production of lung surfactant [67, 68] and inhibit the formation of airway branches and lung buds [28]. This suggests that AMH may inhibit maturation of the lungs in male fetuses and indicate that AMH may be one of the factors causing lung development to be slower in male fetuses [69]. These findings have yet to be confirmed at physiological levels or within the AMH deficient mouse strain, but they indicate that AMH may have a hormonal function as a negative regulator of maturation in utero.

1.6.5 Fertility

1.6.5.1 Follicle regulation

In contrast to males who produce sperm cells throughout their lifetime, females are born with a finite number of oocytes which are exhausted over their reproductive years by ovulation. Oocytes are packaged in follicles and reside in a primordial state until they are selected to
mature into a follicle ready for ovulation. This maturation, or folliculogenesis, is lengthy (taking around 375 days in women) and the number of follicles used at each ovulation need to be limited if the pool of primordial follicles is to last throughout the reproductive years. Folliculogenesis therefore requires tight regulation.

AMH is expressed in the granulosa cells of maturing follicles in the pre-antral and small-antral [70] stages and inhibits primordial follicles maturation [71-73] preventing too many follicles from maturing into pre-antral stage at one time. At the pre-antral and small-antral stage the follicles wait for recruitment to the pre-ovulatory stage. This final maturation is initiated when a follicle is exposed to high concentrations of follicle stimulating hormone (FSH). Each follicle has a unique threshold of FSH required for maturation and it appears that AMH regulates this threshold; the more AMH the follicle expresses the higher its FSH threshold will be and the less likely it will be to mature [74]. Thus, AMH controls follicular maturation by two mechanisms to control two different stages of follicular recruitment.

AMH knockout mice have more maturing follicles than wildtype mice which drains their primordial follicle pool leading to early cessation of ovulation [75, 76]. If this experiment is extrapolated to women, we could expect that women not expressing AMH would enter menopause earlier. AMH deficient women have not been characterised in the literature, however, a polymorphism in the AMHRII receptor has been identified that associates with early menopause [77].

Serum AMH does not fluctuate with folliculogenesis (Figure 1.2) indicating that the paracrine effects of AMH are distinct from its hormonal effects.
1.6.5.2 Sperm Viability

As well as secreting AMH into the blood, the Sertoli cells also secrete AMH into the seminiferous tubules where it enters the seminal plasma [78]. Seminal AMH concentration is much higher than serum AMH plasma [79] meaning that sperm are exposed to higher concentrations of AMH.

There is some contention about whether seminal AMH levels correlate with sperm count or quality in fresh semen samples [78-81]. However, the survival of sperm for long periods (5-22 hours) outside the body can be improved by incubating in high concentrations of AMH [82]. Limited studies in the medaka fish have also suggested that AMH may regulate the maturation of the sperm producing germ cells in the testes in a similar manner to AMH regulation of follicle maturation in the ovary [83].

Curiously, AMH appears to bind directly to sperm, but AMHRII is not expressed in sperm [50, 84]. In vitro studies have implicated a G-coupled receptor, YWK-II, as the putative receptor for AMH signalling pathway in sperm indicating that AMH may have alternative receptors [85, 86]. It is worth noting, however, that YWK-II binds the N-terminal domain of AMH which is distinct from the active C-terminal domain and is thought to be inactive [12, 13]. This work was not able to be replicated by researchers in this laboratory (Imhoff, personal communication).

It is worth noting that in the AMH knockout mouse strain, AMH-/-, male mice have apparently normal sperm that are capable of fertilization, but sperm flow can be obstructed by the presence of persistent Müllerian ducts [58].

1.7 AMH and cell growth regulation

In addition to inhibiting development in ovarian follicles and causing apoptosis in the Müllerian duct, AMH appears to have other roles in growth regulation.
Various cancer cell cultures studies have shown that AMH signalling regulates cell cycle regulators such as IEX-1S, p50, p65, p16, p107 and p130 to inhibit cell proliferation [29-32, 87-90]. Thus far, AMH has been shown to inhibit the growth of breast [29], prostate [31, 88], endometrial [30], cervical [32] and ovarian [91] cancer cell lines. The AMH doses used in these experiments is well in excess of physiological concentrations found in the blood. However, it is important to note that tissues that produce endogenous AMH (i.e. gonads and neurons) may be exposed to higher AMH concentrations than those found in the blood.

AMH treatment has also been shown, at physiological levels, to prevent ocular melanoma cancer proliferation in cell culture [92] and to improve the efficacy of chemotherapy in preventing ovarian tumour proliferation in mice [93].

The AMH deficient mice do not appear to have a high incidence of cancer. However, knocking out the inhibin proteins in mice results in the mice developing testicular tumours at an early age [94]. This suggests that AMH and inhibin signalling may work redundantly to prevent cancer cell proliferation. Testicular tumours are also associated with persistent Müllerian duct syndrome [95] suggesting that men with low AMH signalling may have a higher risk of cancer. However, PMDS is a very rare condition so this has not been extensively studied and it could be that the Müllerian duct remnants themselves are a cancer risk. Whether circulating AMH associates with cancer progression is unknown and is outside the scope of this thesis.

However, normal growth and homeostasis requires a careful control of cell proliferation and apoptosis. For instance, breast tissue mass increases during lactation and then reduces after weaning. Segev et al have shown that there is 80% decrease in AMHRII receptors found in the rat mammary during lactation compared to pre- and post-lactation levels [89]. AMHRII receptor expression is also low during puberty when breast tissue forms rapidly [89]. This suggests that hormonal AMH may be controlling the timing of normal breast development by inhibiting growth outside puberty and lactation.
In this case, it appears that this temporal regulation of AMH function is mediated by the receptor, but, it is important to remember that the concentration of circulating AMH varies greatly throughout development (Figure 1.1). This thesis will explore the possibility that AMH may associate with childhood growth and development.

### 1.8 Is AMH a hormone?

In summary, AMH meets four out of the five criteria of a hormone. That is, AMH is biosynthesised in the Sertoli and granulosa cells (rule 1) and then secreted into the blood (rule 2). The AMH receptor is found in cells outside the gonads (rule 3) and AMH levels are variable (rule 5), although further study is required to determine the sites of AMHRII expression (and thus AMH signalling) and the factors causing AMH variability are unknown.

These properties of AMH suggest that the AMH in the blood is acting on the AMHRII receptors elsewhere in the body to cause a response of some kind. But, it is unknown what this response is and this potential response cannot be assumed to exist. Therefore, AMH falls short of rule 4. The strongest evidence that AMH is a hormone comes from fetal mouse studies where AMH may be a hormonal regulator of the Müllerian duct, brain and lungs in males. However, AMH continues to be present into the blood in males postnatally and begins to be detected in females at the early stages of puberty for unknown reasons.

The investigations of childhood AMH levels are very limited. It is not known why AMH levels fall during boy development and rise during girl development. Studies in rats suggest that AMH may be a hormonal inhibitor of breast growth, in which case, it may be mediating the timing of breast tissue maturation in girls. However, at the beginning of this PhD project there were no published reports relating to the function of AMH in children.

There are no putative hormonal functions of AMH in adults and the general consensus is that AMH is not a hormone in men and women. AMH levels are very different between the sexes during gestation, childhood and advanced age. However, men and premenopausal women have
similar AMH levels. This suggests that the regulation of AMH production may be more similar in men and women than it is in boys and girls. It also indicates that there may be hormonal functions of AMH in adults that are important regardless of sex.

Of course, it is possible that the production of AMH in the gonads may occur solely to fulfil its paracrine role within the gonads (i.e. regulation of folliculogenesis or steroidogenesis) and its presence in the blood may be the result of leakage rather than secretion.

This thesis therefore investigates the role of AMH in human blood and considers if AMH can be classified as a hormone.
Chapter 2: Location of the AMHRII Receptor

AMH signalling occurs via AMHRII, which appears to be a dedicated receptor for AMH (Section 1.5, page 21). Thus, AMH target tissues are likely to express AMHRII. The location of AMHRII throughout the body has not been extensively studied beyond its roles in Müllerian duct regression and in the gonads. However, its expression is found in cells outside of the gonads such as those of the endometrium [96], breast, [89], prostate[88] and cervix [32] in humans and the neurons [27] and fetal lungs [28, 68] of mice indicating that AMH signalling may occur in a variety of tissues. Additionally, a conditional knockout system designed to knockout a target gene from the granulosa cells using the AMHRII gene promoter unexpectedly removed the target gene expression in the heart, liver, pituitary and tail [97] indicating that AMHRII expression may be more extensive than previously thought.

This chapter will investigate the locations of AMHRII expression throughout the body using an AMHRII reporter mouse in order to identify AMH target tissues for further study.

2.1 AMHRII reporter mouse

Gene reporter systems involve using the promoter region of the gene of interest to control a marker protein (i.e. a protein that can produce a distinctive colour or fluorescence). The promoter region of a gene mediates when and where a gene will be expressed, so that the gene product is expressed in the correct cell at the correct time. Thus, in a reporter system, the marker protein is expressed at the same location as the gene of interest and this allows the cells that express the gene of interest to be identified.

In this chapter, the LacZ Cre-Lox system is used. In the AMHRII reporter mouse (AMHRII+/Cre LacZ +), activity of the endogenous AMHRII gene promoter drives the expression of the Cre recombinase enzyme. This enzyme activates a LacZ transgene inserted elsewhere in the genome by removing a STOP insertion that interrupts the gene. LacZ is driven by a powerful ROSA
promoter and once activated is expressed constitutently in the target cells. The LacZ sequence codes for the Escherichia coli protein β-galactosidase which can be detected using a stain containing Xgal (5-bromo-4-chloro-indolyl-β-D-galactopyranosidase). This system is summarised in Figure 2.4.

One caveat of LacZ staining is that the intensity of the LacZ stain within a cell is not indicative of the amount of AMHRII activation, just that activation has occurred. Once activated, the LacZ gene cannot be deactivated, so LacZ-active cells pass their LacZ activity to their daughter cells. Therefore, cells that express LacZ do so either because they have an active AMHRII gene promoter (and express AMHRII) or because they originate from a cell that had an active AMHRII gene promoter.

The LacZ Cre-Lox system is a useful technique for following cell lineage and also a rapid method for screening for potential sites of AMHRII expression. In this thesis, LacZ staining has been used in a preliminary screen to determine putative sites of AMHRII expression. This approach was used as a rapid route to the human studies described in subsequent chapters.
Figure 2.4. **Schematic of AMHRII reporter system.**

When the AMHRII promoter is inactive (A), no Cre recombinase is expressed and the LacZ gene is inactivated by a STOP sequence. When the AMHRII promoter is active (B), Cre recombinase is expressed and acts on the LoxP sites in the LacZ transgene to remove the STOP sequence and LoxP sites. The uninterrupted LacZ gene can then express its product, the beta galactosidase enzyme. This enzyme catalyses the reaction of X-gal to galactose and creates the blue cytoplasmic dye characteristic of LacZ staining.
2.2 Results

2.2.1 Fetal AMHRII reporter mouse

Potential AMH target tissues during embryonic development were identified by sectioning and staining whole AMHRII reporter mouse fetuses (embryonic day 18).

LacZ staining in the AMHRII reporter mouse involved many tissues of the embryo suggesting that AMHRII signalling may be widespread throughout the body (Figure 2.5B, Figure 2.6B). Consistent with other reports, LacZ staining indicated that AMHRII was present in the lungs (Figure 2.6 B), spinal cord, peripheral ganglia (Figure 2.5B, Figure 2.6B), brain and testes (not illustrated).

There was no LacZ staining in the control mouse except for the lumen of the intestines (Figure 2.5A, Figure 2.6A). This staining was also present in mice that did not have the LacZ transgene indicating that the staining is not the result of transgene leakage, but may be from an endogenous enzyme in the intestinal lumen.

2.2.2 Fetal eye

LacZ staining was found in the retina of the eye (Figure 2.7). This staining appeared to be in the neural tunic layer which is consistent with AMHRII expression occurring in the retinal neurons and the photoreceptors of the eye. There was also staining in the cornea and some stain associated with the lens of the eye. However, large parts of the lens were negative of staining and the lacZ stain in the lens appeared to associate with parts of the tissue damaged during sectioning. This indicates that the LacZ staining in the lens may be a result of LacZ enzyme from other locations accumulating in the folds of the damaged section.
Figure 2.5. LacZ staining in a cross-section of the abdomen of E18 mouse fetuses.

LacZ staining was absent in every tissue of the control mouse except the villi of the intestines (A). In contrast, LacZ staining was widespread in the AMHRII reporter mouse (B). Organs of interest are identified in the labelled diagram (C). "UA" marks the umbilical artery, "CA" the coeliac artery, "Ao" the aorta at the point where it meets the coeliac artery, "DRG" the dorsal root ganglion. Bar represents 5mm.
Figure 2.6. LacZ staining in a cross-section of the thorax of E18 mouse fetuses.

LacZ staining was absent from all organs of the control mouse (A), whereas widespread staining is evident in the AMHRII reporter mouse (B). Organs of interest are identified in the labelled diagram (C). “DRG” marks the dorsal root ganglion, “Ao” the aorta, “E” the esophagus, “PA” the pulmonary arteries, “B” the bronchi. Bar represents 5mm.
Figure 2.7. LacZ staining in coronal sections of the eye in E18 mouse fetuses.
LacZ staining is present in the retina in the AMHRII reporter mouse (A), but not in the LacZ control mouse. Arrowhead marks the cornea which surrounds the retina “R”. “L” marks the lens of the eye and “S” marks the skin covering the fetal eye. Bar represents 0.5mm.

Figure 2.8. LacZ staining in skin of E18 mouse fetus.
Lac Staining was present in the epidermis and hair bulb. Bar represents 0.5mm
2.2.3 Fetal epithelium

The skin of the AMHRII reporter mouse fetus was strongly stained suggesting that AMHRII is expressed in many cells of the skin. This staining appeared to be localised to the hair bulbs, the epithelial and epidermis layers with a minority of cells in the dermis being stained (Figure 2.8). Similar staining was seen in the epithelial layer of the nasal cavity (Figure 2.10), mouth and tongue (not shown) which, like the skin, are derived from the ectoderm layer of the embryo.

LacZ staining was also seen in the epithelial layers of the ducts of the liver, bronchi of lungs (Figure 2.6), bladder (Figure 2.5) and intestine (Figure 2.9, note that some of the intestinal staining is endogenous) which are derived from the endoderm layer of the embryo.
Figure 2.9. Cross-section of intestine in E18 mouse fetuses.
LacZ staining was intense in the epithelium of the mucosal layer “M”, the circular muscle layer “C” and the longitudinal muscle layer “L” of the intestine in the AMHRII reporter mouse (A). The control mouse exhibited endogenous enzyme activity in the mucosal layer. “SM” marks the submucosal layer. Bar represents 0.3mm.

Figure 2.10. LacZ staining in a coronal section of the nasal cavity of E18 mouse fetuses.
Staining was present in the epithelium of the nasal sinus cavity in the AMHRII reporter mouse (A), but not in the control mouse (B). “T” marks the turbinate projections of the sinuses, “S” the septum that separates the left and right nasal sinus cavities and “B” some mature bone of the skull. Bar represents 0.5mm.
2.2.4 Fetal muscle

The skeletal muscle in the fetal AMHRII reporter mouse was predominantly free of staining (Figure 2.5, Figure 2.6). There was little, if any, staining in the immature muscle fibres, but staining was associated with tendons (Figure 2.13), including intramuscular tendons, and possibly parts of the intramuscular connective tissue.

In contrast, the smooth muscle layers of the intestine, bladder and esophagus were strongly stained suggesting that AMHRII expression may occur extensively in the smooth muscle cells of the gastrointestinal and urinary systems (Figure 2.5, Figure 2.6).

The cardiac muscle in the ventricles of the heart appeared to be mostly negative of LacZ staining (Figure 2.11). The endothelium and mesothelium layers of the ventricle tissue were stained indicating that, like the other organs studied above, LacZ staining is occurring in the epithelial layers of the heart. The valves and atriums of the heart were stained indicating that AMHRII expression may occur in some cardiac muscle. The blood within the atrium was not stained indicating that AMHRII is not expressed in the cells of fetal blood.

Like the gastrointestinal tracts and the bladder, the smooth muscle cells of the tunica media layer was stained in all blood vessels identified indicating that AMHRII expression may occur throughout the smooth muscle cells of the body.
Figure 2.11. LacZ staining in fetal heart.
Enlargement of Figure 2.6. Arrowhead points to a heart valve, “A” marks the atrium wall, “E” the endothelium, “C” the cardiac muscle of the ventricle, “M” the mesothelium and “B” blood. Bar represents 1mm
2.2.5 Fetal bone

LacZ staining was seen in most, if not all, cells in the cartilage found throughout the body such as the metatarsals of the foot and the growth plate of the femur (Figure 2.12, Figure 2.13). Staining was found in both articular cartilage of joints and the growth plates of bones suggesting that AMHRII expression may occur in hyaline cartilage during bone and joint development (Figure 2.13). There was no staining in mature bone. However, the brittle nature of calcified bone meant that it suffered damage during the sectioning process, so it is possible that the LacZ enzyme may have been lost during processing.

2.2.6 Fetal visceral organs

The liver was free of LacZ staining except for the epithelial layer of the liver ducts (Figure 2.5). Dense LacZ staining was observed in the kidney, adrenal gland, spleen and pancreas which appeared to involve every cell in these organs. In the kidney the staining was strongest in the tubules. Some staining was observed outside of the tubules which may have resulted from the diffusion of the LacZ enzyme into this part of the tissue. In the adrenal gland, the staining seemed to be more intense in the adrenal medulla, although the difference between the adrenal medulla and adrenal cortex was marginal. Staining was also uniform across the spleen and pancreas.
Figure 2.12. LacZ staining in cross-section of a foot in E18 mouse fetuses.
LacZ staining was present in the skin and cartilage of the metatarsals in the AMHRII reporter mouse (A), but not the control mouse (B). "MC" marks some metatarsal cartilage and "MB" some metatarsal bone. Bar represents 1mm.

Figure 2.13. LacZ staining in the growth plate of E18 mouse embryos.
Staining was evident in the growth plate and articular cartilage in the AMHRII reporter mouse (A), but not in the control mouse (B). "C" articular cartilage, "G" growth plate, "B" bone. The arrowhead marks a tendon. Bar represents 1mm.
2.2.7 Adult AMHRII reporter mouse

LacZ reporter expression is constitutive once activated, so LacZ expression observed in fetal cells should be expressed in the adult equivalent of these cells, if the adult cell is a descendant of the fetal cell. LacZ staining was seen in the Sertoli cells and Leydig cells of the testes, and in the neurons of the spinal cord in adult AMHRII reporter mice. No stain observed in the fibre tracts of the central nervous system indicating that most, if not all, of the spinal cord glia were negative. This indicates that LacZ staining is accurately indicating sites of known AMHRII expression in the adult and is in agreement with the fetal results for these tissues.

Consistent with the staining in the fetal reporter mice, no staining was seen in the hepatocytes of the liver or skeletal muscle fibres of the adult reporter mice. However, a comprehensive survey of all skeletal muscles was not performed.

In both sexes, the tunica media layer was stained in every major blood vessel attached to the heart in the adult reporter mouse, but not control mouse (Figure 2.14, Figure 2.15). Some staining was also seen in the coronary arteries. This is in agreement with the staining seen in the fetal reporter mouse. Staining was also seen in the cartilage of the trachea in the adult reporter mouse indicating that the staining seen in fetal cartilage persists (Figure 2.14). Like the fetal AMHRII reporter mouse, the retina of the eye was also stained in adult reporter mouse.

However, LacZ staining was not as widespread in the adult AMHRII reporter mice as it was in the AMHRII reporter fetus. In both sexes, the pancreas and adrenal glands did not show LacZ staining in the adult AMHRII reporter mouse. The kidney, spleen, intestines and skin (hair bulbs only) showed similar LacZ staining in both the reporter mouse and the control indicating the presence of endogenous enzyme that made the results inconclusive. The adult female reporter mouse had weak staining in the lungs, which did not appear to be present in the male, suggesting that the some male lung cells may have been removed by AMH signalling. Table 3.2 compares the LacZ staining results for the fetal and adult AMHRII reporter mouse.
Figure 2.14. LacZ staining in the adult AMHRII reporter mouse (female, 43 days old).
Staining was found in the tunic media of the carotid arteries, the cartilage of the trachea (arrowhead) and the epithelial layer of the esophagus. Staining was not seen in the arteries or trachea in the control mouse (not shown), but some faint staining was seen in the esophagus indicating endogenous staining. Bar represents 1mm.
Figure 2.15. LacZ staining in the aortic wall of adult mice.
LacZ staining was prominent in the tunica media of the aorta in the AMHRII reporter mouse (A), some of the adipocytes of the white fat surrounding the aorta were also stained (bottom left corner). LacZ staining was not present in any layers of aortic wall or surrounding fat in the control mouse (B). Objective diaphragm reduced in aperture to provide contrast to illustrate unstained tissue. Bar represents 0.5mm.
Table 3.2. Summary of LacZ staining results for fetal and adult AMHRII reporter mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fetus</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal cord</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Dorsal root ganglion</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Retina</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Testes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Atrium</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Lung</td>
<td>Yes</td>
<td>Yes*</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Liver</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Kidney</td>
<td>Yes</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>Spleen</td>
<td>Yes</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>Yes (with some background)</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>Hair bulb</td>
<td>Yes</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>Epidermis</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cardiac muscle</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Bladder</td>
<td>Yes</td>
<td>Not studied</td>
</tr>
</tbody>
</table>

Tissues where LacZ staining was consistent between fetal and adult AMHRII reporter mice are marked in the blue zone, those that were not are in the red zone of the table. * staining appears to be limited to females.
2.3 Discussion

LacZ staining was extensive throughout the fetal AMHRII reporter mouse indicating that either AMHRII expression is widespread throughout the fetus or that AMHRII expression occurs in cells lines that differentiate into many different tissues which inherit the LacZ staining.

Derivatives of all three early embryonic layers [98]; the ectoderm (e.g. skin and neurons), the endoderm (e.g. visceral organ epithelium and the epithelium of the digestive and respiratory tracts) and the mesoderm (e.g. heart and the smooth muscle of blood vessels, respiratory tract and gastrointestinal tract) exhibited LacZ staining. However, there were also ectoderm and mesoderm derivatives that were not stained such as the glia of the spinal cord (ectoderm) and the skeletal muscle fibres (mesoderm). This indicates that LacZ activation occurred after the early embryonic layers differentiated into cells with more restricted developmental potency.

LacZ staining was also observed in the atria of the heart in fetal and adult AMH reporter mouse suggesting that AMHRII expression may occur in some cells of this tissue. Interestingly, LacZ staining appeared to be localised to the cardiac muscle of the atria with the ventricles largely unstained in the fetus and not stained in the adult AMHRII reporter mice. The LacZ staining in the ventricles of the fetus was intense in the epithelial layers indicating that AMHRII may be expressed in the fetal epicardium and endocardium of the heart. Extensive staining was not seen in the ventricles of the adult AMHRII reporter mouse. However, staining was seen in the coronary arteries which are related to the fetal epicardium by a common precursor cell line, the proepithelium [99]. It is possible that the cell lines stained in the fetal epicardium and fetal endocardium do not persist in adult epithelial layers or that they make up a very small population of the adult heart and have not yet been identified in the adult AMHRII reporter mouse.

LacZ staining also indicated that AMHRII expression may be present in the smooth muscle layers of the gastrointestinal tract, bladder and blood vessels. The adult AMHRII reporter mouse did
not exhibit staining in these layers suggesting that adult intestinal cells may not be derived from the cells stained in the fetal AMHRII reporter mouse intestines. The staining in the tunica media of the blood vessels was present in both fetal and adult AMHRII reporter mice indicating that the blood vessels are one of the more robust results for potential AMHRII expression. Potential hormonal roles for AMH in the blood vessel are investigated in Chapter 3.

The LacZ reporter system also suggested that AMHRII expression occurs in the connective tissue such as tendons, ligaments and cartilage. The connective tissue was not extensively tested in the adult AMHRII mouse, however staining in the cartilage of the trachea was observed in adult mice. Staining was also observed in the growth plate of fetal bones. The growth plate is an important structure for the longitudinal growth of bones. This corroborates the investigation of AMH as a determinant of stature in boys (Chapter 4).

The fetal AMHRII reporter mouse also indicated putative AMH signalling in the epithelial layers of many tissues. This staining was not observed in the adult reporter mouse which raises two possibilities. The first is that false positive staining is occurring in fetal tissues from endogenous enzyme activity, LacZ transgene leakage or stain precipitation. Alternatively, that false negative staining is occurring in the adult tissue from the soluble LacZ enzyme leaving the tissue before fixation or from repression of the LacZ transgene. This is unlikely because tissues known to express AMHRII (i.e. the Sertoli cells of the testes and the neurons of the nervous system) consistently stained in both fetal and adult tissue. However, it is possible that the ROSA promoter that drives LacZ expression in this system is not active in all cell types.

The second possibility is that the cells that are stained in the fetal tissue are no longer present in the adult tissues. Staining in the fetal tissues was most commonly observed in the epithelial part of the organs. Generally, the cells of the epithelial layer have a short life (for instance, the cells of the epithelial layer of the intestine renew every 7 days [100] and the epidermal layer of the skin
replaces itself every 7 weeks in humans [101]). If AMHRII is not expressed in the adult stem cells of the epithelial cells, then LacZ expression would progressively diminish as the mice mature.

It is interesting to note that epithelial cells are not generally perfused by the blood in adults and tend to receive nutrition and signals from cells and substances in direct contact with their cell membrane. This means that epithelial cells are a less likely candidate for direct hormonal signalling than cells that are perfused by the blood. However, in the fetal environment, the epithelial layers of the fetus are exposed to amniotic fluid which allows the diffusion of nutrients, gases and hormones from one site to another without the use of the bloodstream. TGF-β superfamily hormones such as inhibins and activins are found in the amniotic fluid [102, 103]. However, AMH levels are reported to be undetectable in amniotic fluid in humans [104], although this finding needs to be validated with modern AMH tests.

Further study is required to confirm that AMHRII expression occurs in fetal epithelial cells. If this is confirmed, then it raises the possibility that these tissues are hormonal targets of AMH. The roles AMH signalling could have in these tissues are numerous and will not be discussed extensively in this thesis.

For example, LacZ staining indicated that AMHRII expression occur in fetal skin. LacZ staining was not observed in adult skin, this indicates that, if AMH signalling is functional in skin, that it may regulate roles that are important to fetal, but not adult skin. The skin of the fetus is very different to that of adult mice, fetal skin is permeable to fluids, has an epidermis that does not differentiate into stratified layers until late gestation [105] and some structures like the eyelids and earflaps do not fully develop until after birth. Thus it is possible that AMH signalling mediates parts of this skin development. In fact, the other members of the TGF-β superfamily; activin signalling [106], TGF-β signalling [107] and BMP signalling [108], have all been implicated in skin development in the mouse fetus. This makes AMH signalling a unique pathway of the TGF-β superfamily because it has no recognised role in skin development.
LacZ staining corroborated with previous reports that AMHRII expression is found in the fetal lung [28, 68]. AMH signalling at supra-physiological levels has been shown to inhibit lung development in mice (Section 1.6.4, page 28). LacZ staining in adult lungs appeared to be limited to female mice suggesting that the cells expressing AMHRII in the fetal lungs may have been removed during lung development or that the ROSA promoter is largely inactive in adult lung tissue.

AMH levels in the blood are sexually dimorphic during gestation and childhood (Chapter 1). That is, during early life AMH levels are very high in males and not detectable in females. This means that AMH signalling during fetal life occurs only in males. Many of the organs of the body exhibit sexual dimorphism in size or structure. For example, females have a higher number of alveoli in their lungs than males [109]. Some sexual dimorphism throughout the body may be mediated by AMH signalling in early male life, this would need to be investigated by studies of the tissues identified in this preliminary screen, possibly in the AMH−/− mouse.

The results of this study indicate that AMHRII expression may be expressed in many tissues. It is, therefore, important to consider that the presence of the AMHRII receptor does not necessarily indicate that AMH signalling is occurring in these tissues. For example, the LacZ staining in the female fetal AMHRII reporter mouse was indistinguishable from that of the male reporters despite the fact that female fetuses do not express AMH. Similarly, cells of similar lineages may express AMHRII even though only a distinct population of these cells (or perhaps, no cells at all) will be exposed to AMH. For instance, many of the cells in the fetal epidermis may express AMHRII but it may only be the cells in direct contact with the amniotic fluid that interact with AMH. Alternatively, the presence of local factors produced in cells may block AMH signalling.
This study has identified many tissues that may express AMHRII and therefore are putative targets of hormonal AMH signalling. Each of these tissues is worthy of more intensive investigation. This thesis will focus on the blood vessels and growth plate cartilage as putative targets for AMH. Hence, subsequent chapters will investigate associations between circulating AMH levels and blood vessel and growth characteristics in humans.

### 2.4 Recommendations for further research

The LacZ staining results above do not represent a comprehensive survey of AMHRII expression throughout the body, rather, this experiment provided a time efficient method for identifying tissues for further investigation.

The AMHRII reporter mouse system does not have the resolution to prove that AMHRII expression is occurring in a certain cell at a given time point because LacZ expression can be inherited from parent cells. To determine if AMHRII is being expressed in the tissues identified in this preliminary survey (i.e. blood vessels or cartilage) a method that directly measures the expression of the gene (i.e. mRNA sampling with real time PCR) or the production of the protein (i.e. immunohistochemistry with an antibody directed against AMHRII) is needed to validate the LacZ staining results. The latter technique would be preferable in this situation because this can be performed on intact sections of tissue so that AMHRII expression within individual cell types can be determined. Therefore, preliminary immunohistochemistry trials have commenced in order to optimise the use of an AMHRII antibody in mouse tissue. To determine if the tissues studied are targets of hormonal AMH signalling, the tissues will need to be tested for AMH expression to rule out autocrine or paracrine AMH signalling.

The LacZ staining in this chapter represents a brief survey of AMHRII expression in a selection of tissues at two time points (late stage fetus and adulthood). However, further tissues and time
points need to be studied to gain a greater understanding of AMH signalling throughout the body. The LacZ reporter technique could be used to investigate earlier stage embryos and fetuses to determine when LacZ expression occurred for the first time in a cell lineage. Or LacZ staining could be used in very old mice to determine which tissues AMHRII was never expressed in (i.e. are negative for staining).

Validating these LacZ results with immunohistochemistry will be relatively easy for the embryo and fetal tissues because the whole animal can be sectioned and processed in a short amount of time. However, validating LacZ positive tissues in adult organs will be much more labour intensive as organs will need to be processed individually. Also, the results of this chapter suggest that adult AMHRII reporter mice have a higher amount of background LacZ staining that can cause inconclusive results. Therefore, it may be worthwhile to employ an intermediate step where tissues identified as possible AMHRII positive tissue by the LacZ reporter technique could be validated by real time PCR or western blot. This involves lysing the whole tissue and testing for AMH and AMHRII expression. This technique could be used to focus the more intensive immunohistochemistry survey on organs known to express these genes (and the age at which this expression happens) which would identify the individual cell types could subsequently be identified.
Chapter 3: AMH and Blood Vessels

3.1 Introduction

Histological samples from the AMHR II reporter mouse suggest that AMHR II is expressed in the tunica media of all major blood vessels connected to the heart in the adult mouse (Chapter 2). Blood vessels are comprised of four layers; the tunica adventitia (or tunica externa), tunica media, tunica intima and the endothelium. The tunica media is the largest layer in the blood vessel wall. The chief cellular constituent of the medial layer are the vascular smooth muscle cells which are an important structural component imparting shape, size and strength to the blood vessel [110, 111]. The smooth muscle cells also contribute to blood vessel structure by excreting extracellular matrix proteins such as collagen and elastin that make up the rest of the tunica media [112]. These roles, in addition to mediating inflammation and proteolysis of extracellular matrix proteins [113], make the smooth muscle cells an important regulator of vascular homeostasis and remodelling.

Therefore, this chapter investigates whether AMH may have an effect on blood vessel size or pathology.

3.1.1 Physiology of blood vessels

The size, strength and structure of the blood vessels differ throughout the body. Blood is projected from the heart in high pressure pulses which must be attenuated into a gentler and more consistent flow further downstream in order for gas exchange to occur in cells. Thus, arteries which transport blood from the heart need to be strong to withstand a high stress load and elastic in order to dampen pressure changes between heart beats [114]. To achieve this, arteries have a thick tunica media layer that contains large amounts of muscles fibres and elastin.
fibres which impart strength and elasticity, respectively. The muscle fibres in the tunica media are also important for regulating blood pressure and flow. Contraction of this muscular media (controlled by the autonomic nervous system) reduces the diameter of the blood vessel (vasoconstriction) increasing the resistance to blood flow and increases blood pressure, whereas an increase in the blood vessel diameter (vasodilation) has the opposite effect [115].

Arteries that feed directly from the heart, such as the aorta or pulmonary artery (also known as conducting or elastic arteries), are subjected to the strongest blood pulse pressure and have especially high levels of elastin to withstand this. Arteries further downstream from the heart are composed of a larger proportion of muscle fibres (distributing or muscular arteries) and have greater role in blood pressure regulation [116].

Unlike arteries, veins transport blood at a much lower and consistent pressure. The circulation of the blood in the veins is not pumped by the heart. Instead, the return of venous blood to the heart is facilitated by the thoracic pressure changes that occur from movement of the diaphragm during breathing and the mechanical forces of skeletal muscles pressing on the veins during movement. Therefore, veins have a thinner medial layer with fewer smooth muscle cells and elastin fibres than arteries. Veins also contain valves that prevent the back flow of blood, ensuring that venous blood flows in the correct direction which is often against the forces of gravity [115].

### 3.1.2 Vascular remodelling

In addition to responding to short term changes in blood dynamics (haemodynamics), blood vessels must also adapt their shape and size to long term changes in blood pressure or flow. This is achieved by making permanent changes to the structure of the blood vessel wall, termed vascular remodelling. This remodelling requires the degradation of old tissue and its
replacement with new cells and extracellular matrix. The smooth muscle cells have an important role to play in mediating elastin, collagen and muscle content during remodelling of the tunica media [117].

Vascular remodelling can be initiated by changes in body size. Larger bodies have a greater area that must be perfused by the vascular system and therefore require more blood. So, the size of the blood vessels increase as the body grows larger during childhood or adulthood [118-120]. Therefore, body size has a strong association with the diameter of blood vessels size [121, 122].

Vascular remodelling is also required to adapt to the haemodynamic changes brought about by hypertension and atherosclerosis. Consistently high blood pressure, or hypertension, increases wear and tear on the blood vessel walls [123]. Damage to the blood vessel walls may have a compound effect, by inducing an inflammatory response that causes immune cells to invade the vessel walls creating further damage to extracellular matrix proteins. The blood vessel responds to these insults by increasing the replication of smooth muscle cells which causes the medial layer of the blood vessel wall to thicken [124-126]. This generally decreases the size of the lumen and does not increase the external diameter of the vessel.

Blood vessels must also remodel to repair injuries and accommodate for obstructions in blood flow. This is important in the response to atherosclerosis. Atherosclerosis is a condition where plaques comprised predominantly of lipids and immune cells form in the endothelial and intimal layers of artery walls. This is common during normal aging; however, inflammation, hypertension, and high lipid and cholesterol content in the blood exasperate atherosclerotic development [127]. Atherosclerosis is more prominent in parts of vessels with disturbed blood flow and high tensile strength such as the infrarenal aorta, the carotid bifurcation and coronary arteries [128]. Atherosclerotic plaques damage the blood vessel wall and may grow large enough to occlude blood flow. Vascular remodelling can compensate for the presence of plaques by increasing the diameter of the artery [124].
The capability of the blood vessels to remodel and repair diminishes with aging. This is primarily due to a decrease in vascular smooth muscle cells. Thus, in advanced age, the blood vessels become weaker and have less elastin content [116]. This decrease in vascular tone and elasticity may explain why blood vessel diameter tends to gradually expand in advanced age [129, 130]. In contrast to elastin fibres, collagen fibres accumulate in blood vessel walls with age [116]. This can cause arterial stiffness which decreases the ability of arteries to balance out pulsatile pressure leading to an increase in the blood pressure during the heart beat (systolic pressure) and a decrease in the blood pressure between heart beats (diastolic pressure) thus disturbing blood flow and increasing the damage sustained by the blood vessels [114]. A cumulative effect of weaker, stiffer blood vessels and atherosclerosis may explain why there is a high incidence of cardiovascular events in geriatrics.

### 3.1.3 Blood vessel dilation

When vascular remodelling becomes unbalanced or fails to compensate for vascular changes the walls of the blood vessel can become too weak to withstand the stress of the blood flow. This can result in ballooning or dilation of part of a blood vessel.

Localised expansion of a blood vessel, usually an artery, is termed an aneurysm. An aneurysm involves the expansion of all layers of the blood vessel wall. However, the tunica media appears to be the most important layer of the wall in aneurysm progression. Aneurysms exhibit a decreased elastin and collagen content in the aortic wall. This appears to be the result of unbalanced protease activity from the vascular smooth muscle cells and immune cells [131]. Furthermore, the number of vascular smooth muscle cells is reduced in the medial layer of vessels with aneurysms [132, 133]. This may be due to increased p53 mediated apoptosis in the media [134]. These changes to the structure of the tunica media result a blood vessel that is less stiff and has less tensile strength [135]. The weakened blood vessel subsequently stretches and
balloons when strained. Aneurysms can continue to grow until the blood vessel wall ruptures which, if located in a major blood vessel such as the aorta, causes severe blood loss and death.

Atherosclerosis and hypertension are significant risk factors for aneurysms [113, 136, 137] and sites that are prone to atherosclerotic plaque formation and high shear force, such as the infrarenal aorta, are susceptible to aneurysms. The infrarenal (abdominal) aorta is a common site for aneurysms (abdominal aortic aneurysms or AAA) and this thesis will test if AMH levels associate with the size of the infrarenal aorta.

Aneurysms in veins are rare [138]. However, another form of vein dilation, varicosities (or varicose veins), are relatively common [139]. Unlike aneurysms which are localised, varicosities affect large portions of the affected vein. Although it is generally accepted that varicose veins occur due to weaknesses of the vein wall, the primary cause of this weakness in varicose veins remains controversial. Varicose veins appear to have a thicker tunica intima layer due to invasion of vascular smooth muscle cells from the media as well as less elastin content and greater collagen deposits [140-142]. This indicates that varicose veins may involve a dysfunction of vascular smooth muscle cell regulation in the medial layer. However, histological reports regarding the number and quality of smooth muscle cells in varicose veins are conflicting [112, 139-142].

3.1.4 TGF superfamily and blood vessels

AMH is part of the TGF-β superfamily (Section1.5, page 21) whose members have extensive roles in vascular development, vascular smooth muscle cell regulation and atherosclerosis formation.

Development of the cardiovascular system in the embryo relies on signalling from the TGF-β subfamily (all three ligands; TGF-β1, TGF-β2 and TGF-β3) and the presence of the ALK3 type 1 receptor which is used in BMP and AMH signalling [34, 143]. The development of the
cardiovascular system is not well understood because BMP signalling is also vital for differentiation of the embryo prior to blood vessel cell differentiation. So, germ-line gene knockout studies result in dysfunctional embryo development prior to vascular formation [144].

Proliferation of vascular smooth muscle cells is regulated by the activin and BMP subfamilies with activin signalling (specifically Activin A) increasing proliferation [145, 146] and BMP signalling (BMP2, BMP7 and BMPRII) decreasing proliferation and promoting apoptosis [147-150]. Activin and BMP may also have opposing actions in atherosclerosis formation with BMP signalling promoting formation and calcification of atherosclerotic plaques and activin signalling appearing to have a protective role [21, 151-154]. BMP signalling may also play a role in blood pressure regulation. Large chronic doses of BMP4 impairs vasodilation of the blood vessels and induces hypertension in mice [155]. Consistent with this, mutations in BMPRII cause congenital pulmonary hypertension, a condition characterised by vasoconstriction of the blood vessels of the lungs [156].

TGF-β signalling is an important mediator of postnatal vascular remodelling and repair [157]. TGF-β signalling induces the production of extracellular matrix proteins, particularly collagen, in the fibroblasts and smooth muscle cells of the blood vessel wall [36, 158]. This signalling is upregulated in conditions such as hypertension and varicosities [125, 157, 159]. Deregulation of TGF-β causes Marfan’s syndrome and Loeys Dietz syndrome which are both characterised by aneurysms of the ascending aorta early in life [160, 161]. TGF-β is also an inhibitor of atherosclerosis [162, 163].

Currently, AMH signalling is the only subfamily of the TGF-β superfamily that has not been implicated in vascular homeostasis and disease. The abundance of TGF-β superfamily signalling in the vascular smooth muscle cells indicates that the signalling pathways that AMH shares from other members is present in the vascular smooth muscle cell. Indeed, ALK2, ALK3 and ALK6, the type 1 receptors used in AMH signalling, are present in these vascular smooth muscle cells [164].
This chapter will therefore investigate if AMH levels in the blood correlate with measures of vascular health such as blood vessel diameter, blood pressure and atherosclerosis risk factors.

### 3.2 Results

Serum AMH was measured in 153 healthy men aged between 54 and 93 years. The mean AMH concentration was 26.6 pmol/L and ranged from undetectable to 103.5 pmol/L. Most of the men had AMH levels that were similar to those previously described for younger men [17]. In this narrow age range there was no significant decline in AMH levels with increasing age (Figure 3.16). Three of the cohort (aged 69, 80 and 84 years) had undetectable AMH (< 1 pmol/L) suggesting that a minority of geriatric men do not produce AMH, similar to postmenopausal women. The distribution of AMH levels was skewed to the right and not normally distributed (Figure 3.17 A, Skewness = 1.42, kurtosis = 6.34, Shapiro-Wilk test, W = 0.91, p< 0.001).

#### 3.2.1 AMH negatively correlates with Abdominal Aortic diameter

Exterior diameters were available at three sites of the abdominal aorta; mid-infrarenal, distal-infrarenal and suprarenal. Serum AMH concentration negatively correlated with the diameter of the aorta at the distal- and mid- infrarenal sites (r= -0.22, p= 0.006; r= -0.26, p= 0.008, respectively, Figure 3.18A and B) and even stronger with the maximum of the two measurements, max-infrarenal (r= -0.29, p= 0.002, Figure 3.18C) indicating that men with low AMH levels tend to have larger aortic diameters.

The suprarenal site is less flexible than other parts of the aorta and is resistant to change. Consequently, the suprarenal diameter is used as a reference point for measurements. There was no correlation between the reference suprarenal diameter and AMH (r= -0.10, p= 0.24,
Figure 3.18 D) indicating that the relationship between serum AMH and aortic diameter is localised in regions of the aorta where the diameter is dynamic.

The ratio of infrarenal and suprarenal diameters gives an indication of the general shape of the aorta with a high ratio indicating “ballooning” of the infrarenal aorta. The maximum-infrarenal: suprarenal diameter ratio negatively correlated with AMH to low level statistical significance ($r=-0.17$, $p=0.046$, Figure 3.19). The distal-infrarenal: suprarenal and mid-infrarenal:suprarenal ratios showed the same trend, but were not statistically significant ($r=-0.11$, $p=0.22$ and $r=-0.13$, $p=0.12$, respectively).

As AMH appears to be related to widening of the aorta, the max-infrarenal diameter will be reported hereinafter unless results from the other diameters are notable.
Figure 3.16. Serum AMH levels did not correlate with age in mature men.
AMH levels were measured by ELISA in 154 men aged 54-93 years. There was no significant decline in AMH levels with age ($r=-0.12$, $p=0.13$).

Figure 3.17. Distribution of AMH levels in healthy men (A) and men with abdominal aortic aneurisms (B).
AMH levels were measured in 113 healthy men and 69 AAA patients. The black line indicates mean for healthy controls (26.6 pmol/L) on both graphs. AMH levels were significantly lower in AAA patients (23.6 pmol/L) when AMH levels were controlled for age (Kolmogorov-Smirnov Comparison or KS-test, $D=0.21$, $p=0.034$). The distribution of both cohorts is skewed to the right; healthy men, skewness = 1.42, kurtosis = 6.35, AAA cohort skewness = 1.21, kurtosis = 4.49. The numbers on the x-axis are the centre of the frequency bin (e.g. 20pmol/L ± 5pmol/L).
Figure 3.18. AMH levels correlated with abdominal aortic diameter in healthy men. Aortic diameter was measured via ultrasound (4-7MHz) in 139 men aged 54-93 years. AMH levels correlated with aortic diameter at the distal infrarenal site (A, $r=-0.22$, $p=0.006$), the mid infrarenal site (B, $r=-0.26$, $p=0.008$) and with the maximum of the two measurements (C, $r=-0.29$, $p=0.002$). There was no correlation between AMH levels and aortic diameter at the suprarenal reference site (D, $r=-0.10$, $p=0.24$). Black line indicates best fit with linear regression. Max diameter (cm) = 2.17 − (0.004 x AMH (pmol/L)).
Figure 3.19. AMH levels correlated with the ratio of the max-infrarenal and suprarenal aortic diameters in healthy men. The natural logarithm of the suprarenal diameter was subtracted from the natural logarithm (ln) of the max-infrarenal diameter. Black line indicates line of best fit when analysed by linear regression ($r=0.17$, $p=0.046$, $n=139$).
3.2.2 Aortic diameter did not correlate with other testicular hormones

The correlation between AMH levels and aortic diameter suggests that AMH may influence aortic diameter. Alternatively, aortic diameter may correlate with testicular health with AMH acting as a proxy. To determine if other testicular hormones correlate with aortic diameter, inhibin B (InhB) and testosterone were also measured.

Like AMH, InhB is produced in the Sertoli cells of the testes. AMH levels correlated with InhB \((r=0.22, p=0.006, \text{Figure 3.20})\) indicating that 5% of the variation in either hormone may be explained by a common determinant such as Sertoli cell number or function. There was no correlation between InhB and aortic diameter \((r=-0.04, p=0.660; \text{Table 3.3})\) demonstrating that the association between AMH and aortic diameter is independent of general Sertoli cell output.

Testosterone is a steroid hormone produced in the Leydig cells \([165]\). Once secreted into the blood, testosterone is bound to a liver protein, sex hormone binding globulin (SHBG) which controls the amount of free testosterone available for signalling. There was no correlation between aortic diameter and either testosterone \((r=-0.13, p=0.672)\) or a ratio of testosterone:SHBG \((r=-0.02, p=0.787, \text{Table 3.3})\) indicating that the level of testosterone or free testosterone do not associate with aortic diameter. However, testosterone levels vary throughout the day \([166]\) and the time of day blood was taken was not controlled for in this study which may degrade any correlation between testosterone levels and aortic diameter.

Aortic diameter also did not correlate with vitamin D \((r=-0.04, p=0.671)\) which is a non-testicular hormone that may regulate AMH (Chapter 5).
3.2.3 AMH correlated with aortic diameter independent of body size

Larger people require larger aortas by proportion, so, body size is a strong determinant of aortic diameter [122, 129]. As AMH correlates with height in boys (Chapter 4), body size was also considered as a potential confounder in the AMH-aortic diameter correlate.

Aortic diameter correlated with height, weight, body mass index (BMI), body surface area and hip and waist circumferences in the healthy old men, as expected (Table 3.4). AMH correlated with hip circumference with low level significance using Pearson's (parametric) correlation analysis, but this result was not replicated when analysed by Spearman's (nonparametric) correlation analysis (Table 3.5), indicating that this association is not robust.

There was no correlation between AMH and height, weight, BMI or body surface area (Table 3.5) indicating that AMH levels are independent of body size. Of all the body size measurements, the strongest determinant of aortic diameter was body surface area \((r=0.31, p<0.001, \text{Table 3.4})\). In linear regression analysis, AMH was a significant determinant of aortic diameter independent of body surface area (Table 3.7) indicating that body size is not a confounder of the AMH – aortic diameter correlation. Additionally, the strength of the AMH \((r=-0.37)\) correlate was equal to that of body surface area \((r=+0.37)\), but in an opposite direction, indicating that AMH may be as strong a determinant of aortic diameter as body surface area.

There was no correlation between aortic diameter and hip to waist ratio indicating that, unlike body size, body shape may not be a major determinant of aortic size.
Figure 3.20. AMH levels correlate with InhB levels in healthy mature men. The black line indicates linear regression line ($r=0.22, p=0.006$). AMH (pmol/L) = 30 + (0.06 x InhB (pg/ml)).

Table 3.3. Correlations between hormones and max-infrarenal aortic diameter

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>153</td>
<td>-0.30</td>
<td>0.000</td>
</tr>
<tr>
<td>InhB</td>
<td>153</td>
<td>-0.04</td>
<td>0.660</td>
</tr>
<tr>
<td>Testosterone</td>
<td>137</td>
<td>-0.13</td>
<td>0.672</td>
</tr>
<tr>
<td>SHBG</td>
<td>152</td>
<td>-0.06</td>
<td>0.480</td>
</tr>
<tr>
<td>T:SHBG</td>
<td>136</td>
<td>-0.02</td>
<td>0.787</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>113</td>
<td>-0.04</td>
<td>0.671</td>
</tr>
</tbody>
</table>

Correlates were analysed by Pearson’s linear correlation and the $p$ values recorded, statistically significant $p$ values have been underlined.
Table 3.4. Body size parameters correlated with max-infrarenal aortic diameter

<table>
<thead>
<tr>
<th>Aortic diameter</th>
<th>r</th>
<th>p</th>
<th>rho</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>0.22</td>
<td>0.007</td>
<td>0.22</td>
<td>0.007</td>
</tr>
<tr>
<td>Weight</td>
<td>0.31</td>
<td>0.000</td>
<td>0.29</td>
<td>0.000</td>
</tr>
<tr>
<td>BMI</td>
<td>0.25</td>
<td>0.002</td>
<td>0.24</td>
<td>0.003</td>
</tr>
<tr>
<td>BSA</td>
<td>0.31</td>
<td>0.000</td>
<td>0.30</td>
<td>0.000</td>
</tr>
<tr>
<td>Hip</td>
<td>0.31</td>
<td>0.000</td>
<td>0.27</td>
<td>0.001</td>
</tr>
<tr>
<td>Waist</td>
<td>0.31</td>
<td>0.000</td>
<td>0.29</td>
<td>0.000</td>
</tr>
<tr>
<td>Hip:waist</td>
<td>0.11</td>
<td>0.158</td>
<td>0.11</td>
<td>0.181</td>
</tr>
</tbody>
</table>

Correlates analysed by Pearson's correlation (r) and Spearman's rank order correlation (rho) have been compared. Statistically significant p values have been underlined. BMI = body mass index, BSA = body surface area.

Table 3.5. Body size parameters correlated with AMH concentration

<table>
<thead>
<tr>
<th>AMH</th>
<th>r</th>
<th>p</th>
<th>rho</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>0.08</td>
<td>0.312</td>
<td>0.14</td>
<td>0.089</td>
</tr>
<tr>
<td>Weight</td>
<td>0.11</td>
<td>0.171</td>
<td>0.10</td>
<td>0.205</td>
</tr>
<tr>
<td>BMI</td>
<td>0.09</td>
<td>0.257</td>
<td>0.05</td>
<td>0.559</td>
</tr>
<tr>
<td>BSA</td>
<td>0.11</td>
<td>0.171</td>
<td>0.11</td>
<td>0.168</td>
</tr>
<tr>
<td>Hip</td>
<td>0.16</td>
<td>0.044</td>
<td>0.09</td>
<td>0.274</td>
</tr>
<tr>
<td>Waist</td>
<td>0.10</td>
<td>0.243</td>
<td>-0.01</td>
<td>0.938</td>
</tr>
<tr>
<td>Hip:waist</td>
<td>-0.07</td>
<td>0.395</td>
<td>-0.04</td>
<td>0.655</td>
</tr>
</tbody>
</table>

Pearson’s correlation (r) and Spearman’s rank order correlation (rho) have been compared. Statistically significant p values have been underlined. BMI = Body Mass Index, BSA = Body Surface Area.
3.2.4 AMH correlated with aortic diameter independent of cardiovascular risk factors

Aortic diameter increases with cardiovascular disease risk factors such as atherosclerosis risk factors, inflammation and hypertension [136]. AMH levels in men did not correlate with risk factors for atherosclerosis, including low density lipoprotein (LDL), high density lipoprotein (HDL), cholesterol, triglyceride levels and smoking. None of these factors correlated with aortic diameter in healthy men (3.6).

Thirty seven of the men were taking statin medication which is prescribed to lower cholesterol levels. There was no difference in AMH level between those taking statins and those who were not (KS test, D=0.22, p=0.11). These results indicated that AMH levels do not associate with atherosclerosis development. Consistent with this, AMH levels did not correlate with the thickness of the intimal and intimal layer in the carotid arteries of the men (3.6).

C-reactive protein (CRP) is produced in the liver in response to inflammation and positively correlates with aortic size [167]. Nine men had CRP levels exceeding 10mg/L indicating acute inflammation which may indicate a recent infection or injury. AMH levels in these men were unremarkable (range 6pM to 38pM). In the other 144 men, CRP correlated with aortic diameter (r=0.23, p=0.0002), but not with AMH levels (r=-0.12, p=0.168). AMH and CRP were independent determinants of aortic diameter in a linear regression model (Table 3.7).

Excessive blood pressure, hypertension, is another risk factor of cardiovascular disease. Although generally healthy, 47 men reported a history of hypertension. There was no significant difference in AMH levels between men who had a history of hypertension and men that did not (KS test, D=0.18, p=0.217). However, no information was collected on the extent or duration of hypertension in these men which may have improved the analytical approach.
There was no correlation between current blood pressure and AMH levels ($r=0.03, p=0.715$) or aortic diameter ($r=-0.02, p=0.836$). There was also no correlation between the ankle brachial pressure index (ABI), which is a ratio of the blood pressure measurement in the legs and arms, with AMH levels ($r=-0.16, p=0.064$) or with aortic diameter ($r=0.02, p=0.778$).
### Table 3.6. AMH and aortic diameter correlations with atherosclerosis risk factors

<table>
<thead>
<tr>
<th></th>
<th>AMH</th>
<th>Aortic diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( r )</td>
<td>( p )</td>
</tr>
<tr>
<td>LDL</td>
<td>0.09</td>
<td>0.276</td>
</tr>
<tr>
<td>HDL</td>
<td>-0.14</td>
<td>0.095</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.08</td>
<td>0.333</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>0.09</td>
<td>0.271</td>
</tr>
<tr>
<td>Smoking (pack years)</td>
<td>-0.08</td>
<td>0.309</td>
</tr>
<tr>
<td>Intimal medial thickness</td>
<td>-0.14</td>
<td>0.070</td>
</tr>
</tbody>
</table>

Correlations were analysed by Pearson’s linear correlation and \( p \) values recorded. LDL = low density lipoprotein, HDL = high density lipoprotein. Intimal medial thickness measurements are from the carotid artery.
### Table 3.7. Linear regression models for the predication of maximum infrarenal aortic diameter

<table>
<thead>
<tr>
<th>Model #</th>
<th>Model</th>
<th>Predictor</th>
<th>$r^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AMH</td>
<td>-0.30</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>1</td>
<td>0.09</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMH</td>
<td>-0.37</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2</td>
<td>0.22</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BSA</td>
<td>0.37</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMH</td>
<td>-0.36</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BSA</td>
<td>0.39</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age</td>
<td>0.18</td>
<td>0.028</td>
</tr>
<tr>
<td>4</td>
<td>0.29</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMH</td>
<td>-0.35</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BSA</td>
<td>0.40</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age</td>
<td>0.19</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CRP</td>
<td>0.18</td>
<td>0.090</td>
</tr>
</tbody>
</table>

Four linear regression models were created to predict the maximum infrarenal diameter. The partial correlations of the individual predictors are also recorded in the right hand column. BSA = body surface area, CRP = C-reactive protein.
3.2.5 *Abdominal aortic aneurism patients had lower serum AMH*

The results above show that men with low AMH levels tend to have wider aortas. It is therefore interesting to study AMH levels in men with pathologically wide aortas such as those with abdominal aortic aneurisms (AAA). Sixty-nine AAA patients were included in this study. There was no significant difference in age or body size between the AAA patients and the healthy men (Table 3.8).

If AMH is a negative regulator of aortic diameter, then men with AAA would be expected to have low AMH levels. Consistent with this, the average serum AMH level was 23.6 pmol/L (range undetectable to 84.4 pmol/L) which was significantly lower than healthy controls when age was controlled (KS test, D= 0.21, \( p = 0.034 \)). The distribution of AMH levels in the AAA patients was skewed to the left, indicating that people with high AMH levels were under-represented in this cohort (Figure 3.17 B). This suggests that people with low AMH may have an increased risk of developing AAA.

Diabetes (typeII) is protective against AAA formation [168, 169] and there are conflicting reports that AMH levels are altered in carbohydrate disturbances and insulin resistance [170-172]. Thus, diabetes must be excluded as a potential confounder of the AAA-AMH relationship. Nine men in the AAA patient group had a history of diabetes, their AMH levels (mean 30 pmol/L) were not significantly different from AAA men without diabetes (mean 23 pmol/L, t-test \( p=0.21 \)) indicating that diabetes is not a confounder in this study.

Similarly, there was no significant difference in InhB, testosterone, testosterone:SHBG or SHBG levels in AAA patients compared to healthy men indicating that the AAA-AMH relationship is independent of Sertoli cell number and testicular output.
3.2.6 AMH levels in other blood vessel diseases

Analysis of the AMHRII reporter mouse suggests that AMHRII may be expressed in many arteries and veins (Chapter 2). Therefore, a preliminary study was undertaken to investigate if AMH levels may associate with venous disease or other forms of arterial disease.

AMH levels were measured in the blood of 70 healthy men with varicose veins. As seen in the healthy controls, the distribution of AMH concentrations was skewed to the right and not normally distributed (Figure 3.21 A). Men with varicose veins had significantly higher AMH levels than the healthy control cohort (mean = 34.89, KS-test D=0.23, p=0.011). This association was strengthened by controlling for age (D=0.36, p<0.001) suggesting that men with high AMH levels for their age may be at risk of developing varicose veins. However, it must be noted that the varicose vein cohort covers a wide range of ages and body sizes and is not well matched to the healthy controls (Table 3.8, Table 3.9). Additional age-matched controls are being recruited at the writing of this thesis by Dr Yih Harng Chong.

Blood from 70 men with peripheral artery disease were also analysed. These men were well matched to the healthy controls (Table 3.8, Table 3.9). The distribution of their AMH levels was also skewed and not normally distributed (Figure 3.21 B). There was no significant difference (D=0.15, p=0.193) between the AMH levels of the men with peripheral artery disease (mean =24.94 pmol/L) and the normal controls suggesting that AMH levels may not be altered in peripheral artery disease. The distributions of men with AAA, peripheral artery disease and varicose veins are compared to controls in Figure 3.22.
Table 3.8. Participant characteristics for healthy control and abdominal aortic aneurysm groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Abdominal aortic aneurysm</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>153</td>
<td>69</td>
</tr>
<tr>
<td>age (years)</td>
<td>71.4 (54 - 93)</td>
<td>74.3 (54 - 88)</td>
</tr>
<tr>
<td>height (m)</td>
<td>1.75 (159 - 1.92)</td>
<td>1.75 (162 - 192)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>81.7 (55 - 123)</td>
<td>80.0 (59 - 119)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.6 (19.6 - 38.0)</td>
<td>26.2 (20.5 - 38.9)</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>103.5 (82 - 139)</td>
<td>103.8 (82 - 132)</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>98.2 (79 - 138)</td>
<td>101.4 (85 - 131)</td>
</tr>
<tr>
<td>Hip:waist</td>
<td>0.95 (0.81 - 1.11)</td>
<td>0.98 (0.85 - 1.15)</td>
</tr>
<tr>
<td>BSA</td>
<td>1.99 (1.56 - 2.54)</td>
<td>1.96 (1.64 - 2.42)</td>
</tr>
</tbody>
</table>

Participant characteristics were not significantly different between healthy control men and men with abdominal aortic aneurysms (t-test, p>0.05). Mean (range) ± standard error mean. BMI = body mass index, BSA = body surface area.

Table 3.9. Participant characteristics for varicose vein and peripheral artery disease groups

<table>
<thead>
<tr>
<th></th>
<th>Varicose veins</th>
<th>Peripheral artery disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.5 (22-90)</td>
<td>67.3 (40-87)</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.79 (1.51-2.05)</td>
<td>1.71 (1.53-1.84)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>93.7 (61-141)</td>
<td>76.5 (44-110)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.3 (21-46)</td>
<td>26.2 (17-33)</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>107.4 (90-143)</td>
<td>101.6 (88-115)</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>102.8 (81-135)</td>
<td>98.1 (71-113)</td>
</tr>
<tr>
<td>Hip:waist</td>
<td>0.95 (0.85-1.07)</td>
<td>0.96 (0.79-1.06)</td>
</tr>
<tr>
<td>BSA</td>
<td>2.15 (1.70-2.68)</td>
<td>1.96 (1.37-2.36)</td>
</tr>
</tbody>
</table>

Participant characteristics of the peripheral artery group were not significantly different from those of healthy controls (t-test, p>0.05). However, participants in the varicose vein cohort were significantly younger than the controls. Mean (range) ± standard error mean. BMI = body mass index, BSA = body surface area.
AMH levels were measured in 70 men with varicose veins (aged 22-90 years) and 70 men with peripheral artery disease (aged 40-87 years). The distribution of AMH levels in both the varicose vein and peripheral artery disease is skewed to the right (skewness = 1.01 and 1.42; kurtosis = 1.27 and 4.59, respectively). Black line indicates mean for healthy controls presented in Figure 3.17 (healthy control mean = 26.6 pmol/L). The varicose vein cohort had significantly higher AMH levels (mean 34.9 pmol/L) than the controls (KS-test D=0.2289, p=0.011). There was no significant difference in AMH levels between men with peripheral artery disease and healthy controls (KS-test D=0.1530, p=0.193). The numbers on the x-axis are the centre of the frequency bin (e.g. 20pmol/L ± 5pmol/L).
Figure 3.22. Cumulative frequency of AMH levels in health men and men with vascular disease.
The distribution of AMH level in health controls (green) and men with peripheral artery disease (blue) was not significantly different (KS-test D=0.153, p=0.193). AMH levels in men with varicose veins (orange) was significantly higher (KS-test D=0.229, p=0.011) and significantly lower (KS-test D=0.21, p=0.034) in men with AAA (red) compared to controls.
3.3 Discussion

AMH levels were inversely correlated with external infrarenal aortic diameter in healthy men indicating that AMH is a predictor of aortic diameter. This association was independent of other known predictors such as age, inflammation (CRP) and body size indicating that AMH levels are a novel predictor of aortic diameter. AMH levels were as strong a predictor of maximal infrarenal aortic diameter as body surface area which was the strongest known predictor in this cohort (Table 3.7).

If the association between AMH and aortic diameter is causal then AMH signalling may be affecting an aspect of general cardiovascular function or may be affecting the aorta directly. Analysis of the AMHRII reporter mouse indicates that AMHRII is present in the vascular smooth muscle cells of blood vessels suggesting that the wall of the blood vessel may be the site of AMH signalling. These cells are also the site of extensive TGF-β superfamily signalling meaning that the type 1 receptors and downstream signalling pathway required for AMH signalling are also present in vascular smooth muscle cells.

The experiments outlined in this thesis cannot determine the cellular mechanism by which AMH regulates the diameter of the aorta, if, AMH is a hormonal regulator of aortic diameter. However, there are several potential mechanisms in which AMH signalling could affect aortic size.

There are two broad categories of mechanisms. Firstly, AMH may actively cause blood vessels to be narrow either by influencing blood vessel size during development or by inducing a narrowing of the blood vessel after development with vasoconstriction or vascular remodelling. Secondly, AMH may prevent the blood vessel from enlarging or ballooning by maintaining the blood vessel wall or preventing vessel wall damage such as atherosclerosis. These options are not mutually exclusive because AMH may, for instance, be a regulator of blood vessel development and blood vessel maintenance as seen in other members of the TGF-β superfamily (Section 3.1.4, page 61). If AMH levels are stable throughout childhood and adulthood in men,
then it may be that men with low AMH levels were once boys with low AMH levels and that the association between AMH and aortic diameter was created during development.

However, the results of this study support the latter option, that AMH is inhibiting the expansion of the aorta. AMH levels correlate with the maximum infrarenal aortic diameter more strongly than with the individual measurements of the infrarenal aorta (distal- and mid-infrarenal aortic diameters) alone indicating that AMH associates with aortic widening more strongly than aortic size. Secondly, men with low AMH levels were overrepresented in AAA patients indicating that higher levels of serum AMH may be protective against localised pathological enlargement of the aorta.

The association between AMH and aortic diameter or AAA could also be explained by poor vascular health affecting the function of the testes. However, there was no correlation between aortic diameter and InhB which, like AMH, is a Sertoli cell product. This indicates that, unlike AMH levels, Sertoli cell function does not associate with aortic diameter. The Leydig cells of the testes also appear to be unaffected by vascular changes as testosterone levels did not correlate with aortic diameter although diurnal variation in testosterone limits this analysis. Furthermore, InhB and testosterone levels were not altered in men with AAA which illustrates that testicular function is not affected by altered haemodynamics of the aorta. This indicates that testicular health is not a confounder to the associations between AMH levels and aortic diameter and AAA.

Alternatively, the association between AMH and aortic diameter could be explained by a common determinant that affects both the production of AMH and aortic diameter. For example, AMH levels have been shown to associate with insulin resistance and carbohydrate disturbances although conflicting results are reported [170-174]. Also, having type II diabetes is protective against AAA, indicating that diabetes may be a confounder of the AMH-aortic diameter relationship [168, 169]. However, in this cohort there was no relationship between AMH and diabetes suggesting that diabetes is not a confounder in this study. However, direct measures of
insulin resistance such as fasting glucose levels were not measured in this study and a specifically designed experiment is needed to determine what effect, if any, diabetes has on AMH levels in the general population.

AMH levels were also independent of known risk factors for AAA such as age, inflammation, hypertension and lipid levels suggesting that these factors are not confounders to the AMH-aortic diameter correlation. Additionally, these factors were not strong predictors of aortic diameter in this cohort. For example, when AMH levels and body surface area were accounted for, age and CRP levels only marginally improved a linear regression model predicting maximal infrarenal aortic diameter (Table 3.7).

The AMHRII reporter mouse indicated that AMHRII may be expressed in all major blood vessels connected to the heart (Chapter 2). Therefore, it is also possible that AMH affects the size of arteries and veins throughout the body. Consistent with this, AMH levels may be significantly higher in men with varicose veins. The varicose vein cohort has a wider age range than the controls with a greater number of younger men (Table 3.8, Table 3.9). Therefore, the control cohort needs to be expanded with age-matched controls before this preliminary finding can be validated.

However, both AAA and varicose veins are characterised by structural changes in the blood vessel wall that lead to over expansion. Also, some aneurysmal conditions such as Marfan’s syndrome and coronary artery ectasia have a higher incidence of varicose veins [175, 176], suggesting that these two conditions share some common etiology. This raises the possibility that altered AMH levels may lead to either AAA or varicose veins. However, the AMH levels in these diseases were conflicting with AMH levels significantly lower in AAA and significantly higher in varicose veins. This does not disprove the relationship between AMH levels and varicose veins, however, there are likely to be many factors influencing varicose vein progression.
Varicose veins are most prevalent in post-menopausal women, who have no circulating AMH [177]. This may also be evidence that the association between AMH and varicose veins is not robust. However, the latter point does not disprove the association as there are also reports that suggest that varicose veins are more prevalent in women who reach menopause at a later age [178]. These women may have higher AMH levels during their reproductive years because AMH has a role in conserving the ovarian reserve and prolonging the reproductive time span (Section 1.6.5.1, page 28).

The results of this study suggest that AMH may be an endocrine regulator of blood vessel size and that high AMH levels may be protective against AAA. However, the mechanism by which AMH signalling mediates blood vessel size cannot be determined from this study. The AMHRII reporter mouse indicated that AMH signalling may occur in the vascular smooth muscle cells in the medial layer of the blood vessel wall. As these cells are important for the structure of the blood vessel wall, these results suggest that AMH signalling may mediate the strength of the vessel wall and that low AMH signalling may cause the blood vessel to weaken and expand when faced with high stress (such as that faced by the aorta).

AMH shares signalling components with the BMP family that have been implicated in regulation of atherosclerosis [21, 151-154]. Therefore, AMH signalling could be involved in atherosclerosis which is a significant risk factor for AAA [136]. However, AMH levels did not correlate with measures of atherosclerosis and AMH levels were not altered in peripheral artery disease which is an atherosclerotic disease [179]. This suggests that the association between AMH levels and aortic diameter may be independent of the BMP mediated atherosclerosis pathway.

Furthermore, AMH may also be a regulator of the neurons that innervate the blood vessels, which occurs primarily at the tunica media. Therefore, low AMH levels could lead to a poor vascular tone in blood vessels which may result in blood vessel dilation.
AMH concentration is a strong, novel predictor of aortic diameter and is independent of other known determinants, both physical and metabolic. In fact, AMH levels were as strong a determinant as body surface area which was the strongest known predictor in this cohort. This study indicates that AMH may have a hormonal function in vascular homeostasis like the other members of the TGF-β superfamily. More importantly, this study presents the first evidence of an AMH endocrine role in adults.

3.4 Recommendations for further research

The research presented in this chapter suggests that AMH is a hormonal regulator of blood vessel wall strength. However, the cellular mechanism by which AMH signalling may affect the blood vessel wall needs further elucidation.

First of all, it is not known if the AMHRII receptor is expressed in arteries and veins in humans. To remedy this, samples of arteries and veins (possibly attained during surgical correction of AAA or varicose veins, or from post-mortem samples) could be analysed for AMHRII expression with immunohistochemistry (with AMHRII antibody) or real time PCR (testing for AMHRII mRNA) methods. If these experiments were conducted, it would also be prudent to test for AMH expression to exclude the possibility of paracrine or autocrine signalling.

AMH levels associate with AAA and varicose vein formation which are both conditions characterised by structural changes in the blood vessel wall. To determine if AMH affects blood vessel formation, blood vessels from the AMH knockout mouse (AMH−/−) could be histologically examined for changes in smooth muscle cell number, size or structure. The amount of extracellular matrix proteins such as elastin and collagen fibres could also be measured.

It would also be interesting to determine if AMH levels associate with blood pressure as hypertension is a major risk factor for AAA. Also, changes in artery diameter can drive changes
in blood pressure. Blood pressure was examined in this chapter, but only by a single measurement which is known to be unreliable because changes in blood pressure are a natural response to the excitement of being examined (white coat hypertension)[180]. To circumvent this effect, this study could be repeated with ambulatory blood pressures which are collected over a period of 24 hours during the recruit's normal daily routine. Repeated blood pressure measurements in the AMH−/− mouse could also be performed with tail cuff measurements (similar to human measurements) in conditioned mice or a blood pressure telemetry implant could be used to measure blood pressure long term.
Chapter 4: AMH and Growth

4.1 Introduction

This study arose from a preliminary finding made by Prof Ian McLennan and Dr Kirstie Morgan that AMH levels correlated with height in a small group of boys. In this chapter this preliminary analysis is extended to include a larger number of boys and a variety of other hormones. In Chapter 2, the examination of the AMHRII reporter mouse indicated that AMH signalling may occur in the chondrocytes of the fetal bone growth plate. As the growth plate is a key structure for the longitudinal growth of bones, this chapter will investigate the possibility that AMH is a hormonal regulator of bone growth and development.

4.1.1 Postnatal growth

Postnatal maturation in mammals can be divided into four stages; infancy, childhood, juvenility and adolescence [181]. Each stage has distinct developmental landmarks and growth rates. Infancy is defined as the period from birth until weaning when the offspring is dependent on nutrition from their mother's milk. During this time growth is rapid and the infant develops teeth and the ability to regulate its body temperature [182, 183]. Childhood is defined as the period from weaning until when the offspring is capable of its own feeding and care. This is often regarded as a uniquely human stage of development [181, 184]. However, other species, such as mice, have a period after weaning when parental involvement is important for development [185]. The juvenile stage is the period when the individual is no longer dependent on their parents for their feeding and care until the start of sexual maturation (puberty) which marks the beginning of adolescence. Due to the ambiguities in defining the threshold between childhood and juvenility, these two stages will be combined in this thesis to define a period from weaning...
until the start of puberty (hereinafter pre-pubertal stage or childhood). During the pre-pubertal stage the growth rate is low and many organs reach adult size and function. The length of the pre-pubertal stage is often sexually dimorphic with females entering the adolescent stage before males [186]. The adolescent stage is marked a period of rapid growth where the skeleton grows to adult size and the sex organs develop. The adolescent stage ends when adult size is reached and maturation is complete.

### 4.1.2 Human growth

When compared to other mammals, human infancy is relatively short. The caloric requirements of human infants exceed the supply in breast milk at 12 months of age which coincides with the time that humans are able to feed themselves solid food [187]. At this age, human infants are on average 12% of their adult weight which is much lighter than other mammals who, on average, wean their young when they are 37% of their adult weight [188]. Consequently, humans are less mature in terms of mobility, cognition and dentation than other mammals at weaning and therefore must undertake more development during their pre-pubescent stage [181].

Of all mammals, humans have the longest pre-pubertal stage. During this stage, children grow slowly in size, just 6-10 cm per year in height compared to 18-20 cm per year during human infancy [189]. During the pre-pubescent stage there is no difference in height, weight or muscle development between boys and girls [190-192]. However, developmental milestones such as learning to speak, development of handedness (preference to use left or right hand) and eruption of permanent teeth occur earlier in girls [193-196]. This indicates that boys are maturing slower than girls even though they are same size.

Like other mammals, human females enter adolescence earlier than males. The pubertal growth spurt occurs on average at 10 years in girls and 12 years in males, but with wide variation
between individuals of both sexes [190]. The extended pre-pubertal period in boys allows them to attain a taller adult height. Contrary to popular belief, the difference in adult height between men and women is not due to differences in pubertal growth as there is no difference in the amount of height attained during adolescence in human males and females [197].

### 4.1.3 Bone growth

During infancy, pre-pubescence and adolescence, mammals are constantly increasing in size. This is brought about by the growth of the skeleton. Bone growth occurs through the elongation of the epiphyseal plate (growth plate), a section of growing cartilage at either end of the bone, between the diaphysis (middle of the bone) and the epiphysis (ends of the bones). The epiphyseal plate is elongated through proliferation of cartilage cells, or chondrocytes. The epiphyseal plate is divided into three zones, the resting zone, the proliferative zone and the hypertrophic cartilage zone. The resting zone contains a pool of immature chondrocytes which are subsequently recruited into the proliferative zone where they divide rapidly in columns to elongate the bone. At the hypertrophic cartilage zone the columns of cells differentiate into hypertrophic chondrocytes which increase in length and calcify their extracellular matrix (ossification). Blood vessels invade this matrix and the chondrocytes undergo apoptosis completing the process of bone formation, or ossification [198]. Thus, the epiphyseal plate is extending at its epiphyseal end and becoming bone at its diaphyseal end.
When adult proportions are reached, the chondrocytes in the resting zone are exhausted and the whole epiphyseal plate is ossified (epiphyseal fusion), creating a continuous length of bone and irreversibly halting longitudinal growth [199]. In humans, epiphyseal fusion occurs at the end of puberty after the pubertal growth spurt (tibial ossification, girls 12-16 years, boys 14-19 years)[200]. In other animals, such as mice, bones continue to grow beyond sexual maturation (20-30 days in mice) and epiphyseal fusion occurs later in life (tibial ossification around 70 days in mice)[201].

4.1.4 Endocrine regulators of the growth plate

Growth hormone (GH) is an important regulator of pre-pubescent growth. Children with growth hormone deficiency are significantly shorter than their peers, grow slowly (less than 5 cm per year compared to 6-10 cm for normal children) and have delayed bone maturation and puberty [202]. If left untreated, GH deficiency causes dwarfism with adults maturing 4 standard deviations shorter than their expected adult height [203]. GH-deficiency dwarfism is proportional, that is, trunk and limb length are equally reduced.

GH is secreted from the pituitary into the blood where it acts on the liver to increase the production of insulin like growth factor 1 (IGF-1) in the liver. Both GH and IGF-1 are capable of stimulating chondrocytes at all stages of differentiation within the epiphyseal plate [204], however the majority of GH action on growth is mediated by IGF-1[205].

The secretion of GH is pulsatile and therefore a single measurement of GH concentration is not informative, thus in this thesis the GH-IGF axis is analysed by measuring IGF-1 levels [206]. GH pulses are different frequencies between males and females [207]. This leads to a higher IGF-1 level in pre-pubertal girls compared to pre-pubertal boys [208].
In the blood, less than 5% of IGF-1 is in a free state with most IGF-1 in the blood being bound to IGF binding protein 3 (IGFBP-3) which can alter the activity of IGF-1 [209]. Thus, IGFBP-3 levels have also been considered in this thesis.

Thyroid hormone is another key regulator of pre-pubertal bone growth. Like growth hormone deficiency, children with hypothyroidism have significant growth retardation and delayed skeletal maturation [210]. Thyroid hormone increases the size of the proliferative and hypertrophic zones and promotes the maturation of the hypertrophic zone into bone [198, 210, 211]. Thyroid hormone is also able to regulate the production of GH in the pituitary [212]. Thyroid hormone has two forms, triiodothyronine (T3) and its precursor, thyroxine (T4). The conversion of T4 to T3 is catalysed by type 1 5’deiodinase. This enzyme is regulated by energy intake so that T3 levels are low during underfeeding and high during overfeeding [213]. T3 levels therefore provide a link between growth and nutrition and may mediate growth retardation during starvation and the subsequent catch up growth once energy intake increases.

Both T3 and T4 are found in blood and neither form is sexually dimorphic [214]. T4 levels are constant throughout life, whereas T3 levels are high in childhood and decrease with age indicating that the conversion of T4 to T3 is also age mediated [214].

Sex hormones also regulate bone growth. Estrogen and testosterone increase bone growth causing the pubertal growth spurt in girls and boys, respectively [198, 215, 216]. This may be due to direct actions on the epiphyseal plate [217, 218] or through regulation of the GH-IGF-1 axis [219]. The complete ossification of the epiphyseal plate at the end of puberty is mediated by estrogen in both males and females [198, 215, 216, 220]. Estrogen is produced by the aromatization of androgens (including testosterone) which is catalysed by aromatase (also known as estrogen synthetase). Excess or untimely production of estrogens, as occurs in excess aromatase syndrome or precocious puberty, can cause premature closure of the growth plate and permanently stunted height [221-223]. Conversely, individuals lacking aromatase do not
undergo epiphyseal fusion and continue throughout adulthood, reaching extremely tall stature [216, 224].

Estrogens and androgens are at trace levels during childhood and have therefore not been measured in this study.

### 4.1.5 Regulation of pubertal onset

Bone growth is sexually dimorphic. Males spend a greater amount of time in the pre-pubescent stage of growth than females and consequently attain longer bones [197]. Just how male puberty is delayed (or female puberty advanced) is unknown. One possibility is that there is a male hormone that prevents the onset of puberty. During the pre-pubertal period sex hormones such as estrogens and androgens are only present in circulation in trace amounts in males. However, AMH and inhibin B are excreted from the testes at high concentrations for unknown reasons in infant and pre-pubescent males (Figure 4.23). These hormones are only found at significant levels in males at this time and have changes in concentration that coincide with changes in growth stages (Figure 4.23). For instance, AMH is high during the pre-pubescent stage and decreases in concentration as puberty approaches. Therefore, AMH may signal males to remain in the prepubescent stage.
Figure 4.23 Serum levels of AMH and other gonadal hormones in human and mice males during postnatal growth.

Concentrations are plotted as percentage of maximal expression. In humans (A) maximal expression is AMH=824pM (blue), inhibin B=400pg/ml (red), testosterone=29.33nM (grey). For mice (B) maximal expression is AMH=1142pM, total inhibins (A+B) =4.5ng/ml, testosterone=7nM. End of adolescence defined as skeletal maturation, there is no serum AMH data for adult mice. Created with data from [19, 185, 187, 200, 201, 225-238].
4.2 Results: Part (A) AMH and child growth

4.2.1 AMH was sexually dimorphic and highly variable in children

Serum AMH levels were measured in blood from 102 boys and 30 girls aged five and six years. The mean AMH concentration was significantly higher in boys (1033 pmol/L, range 359 pmol/L to 2193 pmol/L) than in girls (mean 13 pmol/L, range 1pmol/L to 53 pmol/L, Figure 4.24). There was no overlap in boy and girl AMH levels, as expected. Like the men in the previous chapter, the distribution of the boys AMH levels was skewed to the right (Figure 4.24B).

Age significantly correlated with AMH levels in boys, with older boys generally having lower AMH levels (Figure 4.25A). This indicated that AMH concentration is decreasing during childhood development which is consistent with AMH expression patterns (Figure 4.23).

However, when individual boys (n=28) donated blood on two occasions, one year apart (first age= 4.95 to 7.15 years, second age=6.01 to 8.00 years), their AMH levels did not change significantly over the course of a year indicating that AMH levels are generally stable in boys of this age (paired Student’s t-test, mean difference = -0.05 nmol/L, p=0.232, Figure 4.25B).

However, the two boys with the highest baseline AMH levels had large decreases in AMH concentration (0.37 nmol/L and 0.91 nmol/L) suggesting that AMH levels may decrease rapidly in some individuals. The correlation between baseline AMH levels and AMH levels one year on were strongly correlated (r=0.74, p<0.0001) indicating that the rank order of the AMH levels in the boys exhibited minimal change.

4.2.2 AMH levels correlated with height and finger length in boys

There was a significant negative correlation between AMH levels and standing height in boys (r= -0.34, p=0.0004, Figure 4.26A), suggesting that AMH may suppress statural growth in children. Weight also correlated with AMH levels in boys (r= -0.26, p=0.01, Figure 4.26B), however, a boy's
weight is not independent from their height because taller boys are heavier than shorter boys. Consistent with this, the association between AMH and weight was not independent of height when analysed by partial correlation (AMH and weight $r=0.05$, $p=0.626$, when height controlled) indicating that weight and height are not independent measures of growth.

Height also associated with age ($r=0.63$, $p<0.0001$) indicating, unsurprisingly, that boys grow taller with age ($\text{Height (cm)} = 76.8 + (6.80 \times \text{Age (years)})$). The association between AMH and height remained significant ($r=-0.21$, $p=0.034$) when age was controlled by partial correlation indicating that age and AMH levels are independent determinants of height (Table 4.10).

Standing height is the product of the growth of many different bones, such as the long bones of the limbs or the vertebra in the spine. To determine if AMH levels associate with measurements of individual parts of the body, the finger length of the boys were also analysed. AMH concentration correlated with the lengths of the second ($r=-0.34$, $p=0.0004$) and fourth ($r=-0.33$, $p=0.0007$) digits of the boy’s hands (Figure 4.27). These associations are similar to that seen for height, indicating that the association between AMH and growth can be detected in localised measurements of the body.
Figure 4.24. AMH levels compared with age in five- and six-year-old children.
AMH was measured in 30 girls (A) and 102 boys (B). As expected boys had significantly higher levels of AMH (Average =1033 ± 39 pmol/L) than girls (13 ± 2 pmol/L) with both groups showing high variability about the mean. The distribution of AMH levels in boys was skewed to the right (skewness 0.62, kurtosis 0.17).

Figure 4.25. AMH levels decrease with age in boys, but remain stable over one year.
AMH significantly decreased with age in a cross-sectional study of 102 boys aged five- and six-years-old (r= 0.30, p=0.002)(A). However, when boys gave blood samples one year apart (B), AMH levels were not significantly different within individuals (paired t-test, p=0.26) and their first and second samples correlated (r=0.74, p< 0.0001) indicating that the rank order of AMH levels was retained over the course of a year.
Figure 4.26. AMH correlated with height and weight in boys.
AMH levels negatively correlated with stature in 102 boys (A, $r=-0.34$, $p=0.0004$). Boy height (cm) = $123.3 - (0.0051 \times$ AMH (pmol/L)). AMH also negatively correlated with weight (B, $r=-0.26$, $p=0.01$). This correlate was not independent of height (AMH and weight $r=0.05$, $p=0.626$, when height controlled).
Table 4.10. Models predicting height in five- and six-year-old boys

<table>
<thead>
<tr>
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<th>Models including InhB</th>
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<tbody>
<tr>
<td></td>
<td>Predictor</td>
<td>Model</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$r$</td>
</tr>
<tr>
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</tr>
<tr>
<td>2</td>
<td>AMH</td>
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</tr>
<tr>
<td></td>
<td>Age</td>
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</tr>
<tr>
<td>3</td>
<td>AMH</td>
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</tr>
<tr>
<td></td>
<td>Age</td>
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</tr>
<tr>
<td></td>
<td>Parental Height</td>
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</tr>
<tr>
<td>4</td>
<td>AMH</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Parental Height</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>IGF-1</td>
<td>0.57</td>
</tr>
<tr>
<td>5</td>
<td>AMH + InhB</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Parental Height</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>IGF-1</td>
<td>0.56</td>
</tr>
</tbody>
</table>

A selection of linear regression models predicting the height of boys were examined. Predictors in the models were also analysed by partial correlation with part correlate and significance also listed. Parental height is mid parental height.
Figure 4.27. AMH levels correlated with finger length in boys.
The length of the second digit (2D) and the fourth digit (4D) were measured on the left and right hands of 5- and 6-year-old boys. The average 2D length (left and right hands) correlated with AMH levels ($r=-0.34, p=0.0004$). Similarly the average 4D length also correlated with AMH levels ($r=-0.33, p=0.0007$).
4.2.3 AMH levels correlated with growth maturity in boys

A boy may be smaller than his peers because he is destined to be a small adult or because his development is occurring at a slower rate. Therefore, AMH levels may associate with the size of a boy or, alternatively, with the rate that the boy is maturing.

Height has high heritability [239] and thus a child’s adult height can be accurately estimated from the height of their parents [240]. To determine if AMH levels may associate with final adult height in the boys, the height of their parents were compared to the boys’ AMH levels. There was no correlation between AMH levels and maternal or paternal heights, even though these parental heights were a strong determinant of the child’s current height (Table 4.2). Mid parental height (mean of maternal and paternal height), which is the best predictor of final height, also did not correlate with AMH concentration ($r=0.03, p=0.770$). These results suggest that AMH levels are not a major predictor of final adult height in boys.

To determine if AMH levels associated with maturity, the current height of the boys was compared to the average of their parents’ heights to calculate the height a boy had achieved at 5-6 years as a proportion of their predicted adult height (relative height). The average relative height in the boys was 0.69, indicating that the height of an average five- or six-year-old boy is 69% of his final adult height (range 60% to 77%).

Relative height correlated negatively with AMH levels ($r=-0.37, p=0.0003$) indicating that AMH levels are low in boys that are growing faster for their age. This suggests that AMH levels are a determinant of maturation rather than adult size.
4.2.4 The correlation between AMH and growth is independent of other growth hormones.

IGF-1 levels were a strong determinant of height in boys ($r=0.51, p=0.000$, Table 4.1 and 4.2) indicating that the growth hormone axis is an important enhancer of boy growth. Relative height also positively correlated with IGF-1 concentration ($r=0.48, p=0.0000$, Figure 4.28C) indicating that the growth hormone axis is a positive regulator of maturation. AMH levels did not correlate with IGF-1 levels (Table 4.1, Table 3.2) and adding IGF-1 to a linear regression model containing AMH did not degrade the significance of the AMH coefficient, indicating that the two hormones are independent predictors of growth.

AMH also did not correlate with T3 or T4 indicating that AMH associates with growth independent of thyroid function (Table 4.2). Interestingly, these hormones were not significant determinants of boy height (Table 4.2) signifying that although these hormones are crucial for correct growth, T3 and T4 levels are not predictive of the amount of growth attained in healthy boys of this age.

AMH levels were also independent of parathyroid hormone levels (Table 4.2) suggesting that AMH levels do not associate with bone turn over or calcium metabolism.
Table 4.11. Determinants of AMH, InhB and Height

<table>
<thead>
<tr>
<th>Determinant</th>
<th>AMH</th>
<th>InhB</th>
<th>Height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>AMH</td>
<td>0.47</td>
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</tr>
<tr>
<td>InhB</td>
<td>0.47</td>
<td>0.000</td>
<td>-0.29</td>
</tr>
<tr>
<td>IGF-1</td>
<td>-0.01</td>
<td>0.930</td>
<td>-0.10</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.06</td>
<td>0.940</td>
<td>-0.16</td>
</tr>
<tr>
<td>IGF-1/IGFBP-3</td>
<td>0.11</td>
<td>0.330</td>
<td>0.16</td>
</tr>
<tr>
<td>PTH</td>
<td>-0.17</td>
<td>0.137</td>
<td>-0.07</td>
</tr>
<tr>
<td>T3</td>
<td>0.03</td>
<td>0.775</td>
<td>0.00</td>
</tr>
<tr>
<td>T4</td>
<td>0.07</td>
<td>0.533</td>
<td>-0.09</td>
</tr>
<tr>
<td>Maternal Height</td>
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<td>0.371</td>
<td>0.06</td>
</tr>
<tr>
<td>Paternal Height</td>
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<td>0.670</td>
<td>-0.03</td>
</tr>
<tr>
<td>Mid-parental Height</td>
<td>0.03</td>
<td>0.770</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Correlations for various predictors of AMH, InhB or height from Pearson’s correlation analysis. Statistically significant values are underlined.
Figure 4.28. The levels of AMH (A), InhB (B) and IGF-1 (C) in five- and six-year-old boys correlated with their height relative to the height of their parents. Black line indicates line of best fit when analyses by linear regression; AMH, $r=-0.37$, $p=0.003$, $n=92$; InhB with outlier (clear), $r=-0.35$, $p=0.008$, $n=88$, InhB without outlier $r=-0.34$, $p=0.001$; IGF-1, $r=0.48$, $p=0.0001$, $n=82$. 
4.2.5 Inhibin B also correlated with growth measures

AMH and Inhibin B (InhB) are both products of the Sertoli cell and their levels correlated in boys ($r=0.47$, $p=0.000$) indicating that Sertoli cell number or performance accounts for around 22% of the variation in the concentration of either hormone. Like AMH, InhB also correlated negatively with height ($r=-0.29$, $p=0.004$, Figure 4.29) and relative height ($r=-0.35$, $p=0.008$, Figure 4.28B) in the boys. In linear correlation models InhB and AMH reduced each other’s partial correlation with height (AMH $r=-0.21$, $p=0.04$; InhB $r=-0.18$, $p=0.09$), but did not obliterate it. Indicating that part of the association between AMH and height is independent of InhB, and vice versa.

When InhB was exchanged for AMH in linear models including age, parental height and IGF-1, InhB had very similar $r$ values to AMH (Table 4.10). The correlation between AMH and InhB is less than 25% and is insufficient to explain why both AMH and InhB would correlate with height with similar strength. This suggests that AMH and InhB may be redundant regulators of height in boys. If so, the combined effect of AMH and InhB should relate to height more strongly than either hormone alone. To test this idea statistically AMH and InhB were each normalised to their mean concentration and then added together. Consistent with this, the sum of the normalised hormones correlated to height ($r=-0.35$, $p<0.0001$) more strongly than either hormone alone.
Figure 4.29. Height correlated with serum InhB in five- and six-year-old boys.
InhB levels were measured by ELISA in 96 boys. Black line is best fit with linear regression, with outlier (clear) $r=0.29$, $p=0.004$, without outlier $r=0.21$, $p=0.037$. 
4.3 Part (B) AMH and mouse growth

If the relationship between AMH and height in boys is causative, then AMH may be affecting growth during childhood, possibly through the AMH receptor found in bone (Chapter 2). However, the effects of AMH are inseparable from those of InhB within correlative data. Therefore, AMH knockout (AMH−/−) mice were used to determine the effects of AMH alone. AMH−/− mice are missing the AMH gene and have persistent Müllerian duct syndrome, but have normally functioning testes, testosterone, Sertoli cell number [58, 241]. Leg and tail samples were collected from inbred AMH−/− and AMH+/+ mice stored in the lab tissue bank and scanned in a high resolution μCT scanner. The images of the bones (tibia, metatarsals and caudal vertebra) were then measured in silico for length, width and density (Figure 4.30).

4.3.1 Bone length was not significantly reduced in young AMH−/− mice

The human correlative data suggests that AMH may slow maturation in five- and six-year-old boys, but have little effect on their adult size. To determine if a similar relationship is present in mice, the bones of 20-day-old male mice were scanned. Mice at this age are weaned from their mother’s milk, but have not yet reached sexual maturation and have not completed bone growth. The growth plates of the bones were visible indicating that all the bones were still in the process of elongating at 20 days. The diaphysis, the length of the bone not including the growth plates or caps on either end, were able to be measured accurately to give a measurement of the amount of mature bone growth achieved at this age. There was no significant difference in the length of the bone or diaphysis of the tibia or vertebra although there was a trend for the AMH−/− to have shorter (2.5%) tibia (Figure 4.31, Figure 4.32). Similarly, there was no significant difference in metatarsal length or diaphysis length in males of this age (Table 4.12).
Figure 4.30. Measurements of the tibia in mice.
A three dimensional image of the tibia was created by μCT scanning of the leg of the mouse. The tibia was then viewed on the coronal (A, yellow line), transverse (B, purple line) and sagittal (C, blue line) planes. Green lines indicate tibia length measurements.

Figure 4.31. Tibia measurements are not significantly different in young AMH+/− mice.
The tibia of the right leg was measured by μCT in 20-day-old male mice. There was no significant difference in the length (A) or width (B) of the tibia (Students t-test, p=0.31 and p=0.25, respectively) between AMH+/+ (n=6, blue) and AMH+/− (n=6, green) mice. The length of the two epiphyseal plates and epiphysis were also measured (C), AMH+/− mice tended to have smaller epiphyses, but this different was not significant (p=0.07).
Figure 4.32. Vertebra measurements are not significantly different in young AMH+/− mice. The fourth caudal vertebra of the tail was measured by µCT in 20-day-old male mice. There was no significant difference in the length (A) or width (B) of the vertebra (Students t-test, p=0.60 and p=0.80, respectively) between AMH+/+ (n=6, blue) and AMH−/− (n=6, green) mice. There was also no significant difference in the length of the two epiphyseal plates and epiphysis (p=0.53, C).

Table 4.12. There was no significant difference in metatarsal length in young AMH−/− male mice

<table>
<thead>
<tr>
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<th>AMH+/+</th>
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<th>AMH−/−</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>M3 length</td>
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<td>6.42</td>
<td>0.134</td>
</tr>
<tr>
<td>M3 length diaphysis</td>
<td>5.68</td>
<td>0.030</td>
<td>5.61</td>
<td>0.124</td>
</tr>
<tr>
<td>M2 length</td>
<td>6.23</td>
<td>0.030</td>
<td>6.18</td>
<td>0.110</td>
</tr>
<tr>
<td>M2 length diaphysis</td>
<td>5.48</td>
<td>0.031</td>
<td>5.44</td>
<td>0.105</td>
</tr>
<tr>
<td>M4 length</td>
<td>6.51</td>
<td>0.029</td>
<td>6.4</td>
<td>0.105</td>
</tr>
<tr>
<td>M4 length diaphysis</td>
<td>5.73</td>
<td>0.029</td>
<td>5.65</td>
<td>0.123</td>
</tr>
</tbody>
</table>

M3 = third metatarsal, M2 = second metatarsal, M4 = fourth metatarsal. Diaphysis length is the length of the bone without the growth plates. SEM= standard error mean.
4.3.2 Early growth was not affected in AMH deficient mice

The data from the child growth study indicate that AMH may slow maturation rate. If this relationship is also true in mice then AMH\(^{-/-}\) mice should grow faster than AMH\(^{+/-}\) mice, but reach the same adult size.

To investigate longitudinal growth in AMH\(^{-/-}\) mice, litters from AMH\(^{+/-}\) x AMH\(^{+/-}\) matings were reduced to four pups to control for litter size variation in pup growth and to prevent competition between siblings. The litters were comprised of as many male pups as possible. Pups were observed and weighed at birth and then daily from 7 days old until over 30 days old. All pups opened their eyes for the first time at 12 or 13 days of age and all pups lost weight at 15-16 days of age consistent with the onset of natural weaning [242, 243]. There was no significant difference between AMH\(^{-/-}\) and AMH\(^{+/-}\) mice in the timing of these milestones.

Pup weight had high variation between litters. To account for inter-litter variation a model was fitted to the data which included litter groups as a variable. Mouse growth was best fitted to a quintic model (Figure 4.33), including AMH genotype in the model did not improve the model, nor was the genotype coefficient significant (\(p=0.539\)). When models were built for each genotype group (AMH\(^{-/-}\), AMH\(^{+/-}\) and AMH\(^{+/-}\)), the models were very similar and there was no significant difference between the coefficients of the model indicating that genotype did not have a significant effect on mouse growth across the data.

The quintic model fitted the data well however, some parts of the data such as the decrease in the pup’s weight at weaning and separation from their parents were no accurately depicted in the modelled line (Figure 4.33). Time points of specific interest such as natural weaning at 16 days, separation of pups from their parents at day 20, or the start (day 1) or peak growth rate (day 29) were tested individually by Student’s t-test and did not show significant differences in pup weight between genotype groups (Table 4.13).
Growth periods such as puberty are marked by rapid changes in growth rate and do not necessarily occur when an animal reaches a specific age. To determine if AMH may affect growth rate, the growth rate of each mouse was collected for each day and a model created for each genotype group (Figure 4.34). The models for each group were very similar until day 17 where the AMH+/− curve diverged to a faster growth rate than the other genotypes. The AMH−/− growth rate curve did not reach as high a peak growth rate as the other genotypes. However, there was no significant difference between the coefficients of the models and a model created for the entire group was not improved with the addition of genotype.
Figure 4.33. Weight of mice from AMH+/− x AMH+/− matings during early development.
Litters were reduced to four pups, all male if possible, and pups were weighed daily from 7 days of age (A). Male pups have been grouped by genotype AMH+/− (dark blue), AMH+/− (light blue) and AMH−/− (broken green lines). Mouse growth was best modelled with a quintic function (B) centred on age 16 days (AgeC = Age-16) and taking litter group into account (AIC = 1256, p=0.000). The equation for this model is weight(g)=8.66 + (0.1897 AgeC) - (5.42E-3 AgeC^2) + (2.97E-3 AgeC^3) + (3.55E-5 AgeC^4) - (6.77E-6 AgeC^5). Adding the genotype variable did not improve the model (AIC=1260), and the genotype coefficient was not significant (coefficient = 0.05, p=0.539) indicating that there is no effect of genotype on weight in early mouse development.
Figure 4.34. Daily growth rate in mouse pups was not affected by their AMH genotype. Mouse growth data from Figure 4.33 has been presented as daily weight increase. Individual growth rates for each mouse have been averaged. Mouse growth rate was best modelled using a quintic function. Age was centred at 17 days (Age\(_c\)=mouse age-17) and litter group have been taken into account (Akaike information criterion (AIC)=354). The equation for this model is growth rate (g/day)=0.12 + (0.004 Age\(_c\)) + (0.015Age\(_c^2\)) – (0.0002 Age\(_c^3\)) – (0.0001 Age\(_c^4\))+(0.000003 Age\(_c^5\)). Adding AMH genotype to the model did not improve the model and there was no significant effect of genotype (AMH\(\sim\) 0.009 times smaller than wildtypes \(p=0.69\), AMH\(\sim\) 0.1 times bigger than wildtypes \(p=0.63\)).
Table 4.13. Weight of AMH+/− and AMH−/− mice compared to AMH+/+ mice at development milestones

<table>
<thead>
<tr>
<th></th>
<th>AMH+/+</th>
<th>AMH+/-</th>
<th>AMH−/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>n</td>
</tr>
<tr>
<td>Birth (day 1)</td>
<td>1.53</td>
<td>0.03</td>
<td>15</td>
</tr>
<tr>
<td>Weaning (day 16)</td>
<td>8.50</td>
<td>0.43</td>
<td>11</td>
</tr>
<tr>
<td>Separation (day 20)</td>
<td>9.69</td>
<td>0.47</td>
<td>9</td>
</tr>
<tr>
<td>Peak growth (day 29)</td>
<td>14.76</td>
<td>0.58</td>
<td>10</td>
</tr>
</tbody>
</table>

Weight (g) of mice at birth, onset of natural weaning, separation from their parents and during peak growth. SEM= standard error mean, p values are from t-test compared to AMH+/+. 
4.3.3 Adult bone length was reduced in AMH−/− mice

To determine if AMH affected adult size, the tibia, vertebra and metatarsals from adult (140 days old) AMH+/+ and AMH−/− mice from both males and females were measured. As could be expected, males had longer bones than females. There was no interaction between sex and AMH genotype in the length or width of any of the bones when analysed by two-way ANOVA.

Male AMH−/− mice had significantly shorter tibia lengths than male AMH+/+ mice (p=0.01, students t-test, Figure 4.35A) indicating that AMH may promote longitudinal bone growth. Similarly, female AMH−/− had significantly shorter tibias than female AMH+/+ mice (p=0.03). The decrease in tibia length in the male (0.6mm) and female (0.4mm) AMH−/− was similar indicating that the loss of AMH expression affects both sexes in a similar way. Furthermore, when analysed by two-way ANOVA, sex and AMH explained the same amount of variation in tibia length (27.4%, p=0.001, for both) suggesting that the effect of AMH on bone length is as large as the effect of sexual dimorphism. Consistent with this, the tibia lengths of the AMH−/− male mice were similar to that of the AMH+/+ female mice.

The effect of AMH on bone length was also seen in the caudal vertebra. Male AMH−/− mice had significantly shorter vertebra than male AMH+/+ (p=0.003, Figure 4.36A). There was no significant difference in vertebral length between female AMH−/− and AMH+/+ although AMH−/− females showed a trend to be smaller. Like tibia length, vertebra length in AMH−/− males was not significantly different from AMH+/+ females indicating that the size of the AMH effect was similar to the effect of sexual dimorphism. This was confirmed by two-way ANOVA (Sex 14.4%, p=0.03, AMH 17.6, p=0.02).

The first metatarsal of the second (M2), third (M3) and fourth (M4) digit was measured. Male AMH+/+ mice had longer metatarsals than female AMH+/+ mice, but this difference was only significant in M2 (t-test p0.041). There was no significant difference in metatarsal size in AMH−/− mice of either sex, but there was an overall trend for the mutants to be smaller (Table 4.14).
There was no effect of AMH on the width of the tibia (Figure 4.35B), vertebra (Figure 4.36B) or the metatarsals, indicating that AMH promotes the growth of bones in length, but not in width which occurs by a different mechanism.

4.3.4 AMH does not affect bone density

To determine if bone maintenance is different in AMH−/− mice, bone densities of the tibia, vertebra and metatarsals were measured. There was a higher amount of variation in bone density than in bone length or width (Figure 4.37). Bone density was sexually dimorphic in the caudal vertebra, with females having higher densities than males ($p=0.001$). There was no significant difference in bone density between AMH+/+ and AMH−/− in any of the bones studied, suggesting that AMH regulates bone growth rather than bone maintenance.
Figure 4.35. Tibia length was significantly shorter in adult AMH<sup>-/-</sup> mice.
Tibia length and width was measured by µCT in AMH<sup>+/+</sup> and AMH<sup>-/-</sup> mice that were 140-day-old. Male AMH<sup>-/-</sup> mice (n=9, blue) had shorter tibia lengths than male AMH<sup>+/+</sup> mice (n=7, green, #1 p=0.01, t-test), but tibia width was unaffected (p=0.33). Similarly, female AMH<sup>-/-</sup> mice (n=7, purple) had shorter tibia than female AMH<sup>+/+</sup> mice (n=6, pink, #2 p=0.03), but not wider tibia (p=0.36). Both tibia length and width were sexually dimorphic (#3 p=0.03 and #4 p=0.03, respectively) with males being larger than females. There was no interaction in effect between AMH and sex on tibia length when analysed by two-way ANOVA (p=0.667).
Figure 4.36. Adult AMH⁻/⁻ male mice had significantly shorter caudal vertebra.
The fourth vertebrae of the tail (caudal) was measure by µCT scan in male and female AMH⁺/+ and AMH⁻/⁻ mice that were 140-day-old. Male AMH⁺/⁻ (n=9, blue) mice had shorter vertebral lengths than AMH⁺/+ mice (n=7, green, #1 \( p = 0.003 \), Student's t-test), but vertebral width was not significantly different (\( p = 0.67 \)). There was no significant difference in vertebral length and width between female AMH⁻/⁻ mice (n=7, purple) and female AMH⁺/+ mice (n=6, pink, \( p = 0.44 \) and \( p = 0.12 \), respectively). Vertebral length was different between AMH⁺/+ males and females (#2 \( p = 0.01 \)), but vertebral width did not (\( p = 0.89 \)). There was no interaction in effect between AMH and sex on vertebra length when analysed by two-way ANOVA (\( p = 0.274 \)).

Table 4.14. Metatarsal length of 140-day-old mice

<table>
<thead>
<tr>
<th></th>
<th>Male AMH⁺/+</th>
<th>Male AMH⁻/⁻</th>
<th>Female AMH⁺/+</th>
<th>Female AMH⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>M2 Length</td>
<td>7.00 ± 0.059</td>
<td>6.91 ± 0.035</td>
<td>6.85 ± 0.055</td>
<td>6.68 ± 0.040</td>
</tr>
<tr>
<td>M3 Length</td>
<td>7.20 ± 0.055</td>
<td>7.10 ± 0.013</td>
<td>7.14 ± 0.054</td>
<td>6.94 ± 0.051</td>
</tr>
<tr>
<td>M4 Length</td>
<td>7.28 ± 0.066</td>
<td>7.28 ± 0.028</td>
<td>7.24 ± 0.101</td>
<td>7.01 ± 0.057</td>
</tr>
</tbody>
</table>

M2 = second metatarsal, M3 = third metatarsal, M4 = fourth metatarsal, SEM= standard error mean. \( p \) values from Student's t-test compared to AMH⁺/+ are listed.
Figure 4.37. AMH had no significant effect on bone density.
Bone density was measured with a µCT scan in the tibia, fourth caudal vertebra, metatarsals (2nd to 4th) and the medial cuneiform of the foot in 140-day-old mice. Male AMH+/- (blue), male AMH-/- (green), female AMH+/- (purple) and female AMH-/- (pink) mice were compared. There was no significant difference in bone density in any of the bones studied between AMH+/- and AMH-/- mice in either sex. Vertebral bone density was significantly higher in AMH+/- females compared to AMH+/- males (#1 \( p=0.001 \), two way ANOVA).
4.4 Discussion

4.4.1 AMH may retard the development of boys

AMH levels inversely correlated with the height of boys and with the length of their fingers indicating that AMH levels associate with either the adult size a boy will reach or how much of his adult size he has achieved. Consistent with the latter, AMH and InhB did not correlate with the average height of their parents which is an estimate of a boy's adult size. This is supported by other studies which have found no correlation between adult size and adult AMH levels [81]. However, longitudinal studies are needed to confirm this.

The ratio between a boy's height and his parents' heights (relative height) is an index of how far he has progressed towards his adult height. The boys' chronological age positively correlated with this ratio, whereas AMH levels had the opposite effect to age, suggesting that AMH retards maturation. Thus, boys with higher AMH levels appeared to be shorter because they were maturing more slowly than their peers. It is worth noting that AMH signalling has been implicated as a regulator of lung maturation (Section 1.6.4, page 28) indicating that AMH may control the maturation of multiple tissues.

AMH levels varied considerably between the boys which may explain why the maturation rate of boys is highly variable [189, 244, 245]. If AMH is a causal regulator of growth, then the average effect of any serum concentration of AMH can be estimated from the linear regression line in Figure 4.26. The difference between the boys with the highest and lowest levels of AMH was 1.7 nmol/L. This creates a calculated height difference of 9.3 cm over the range of AMH levels seen for this narrow age range. This is the equivalent of over a year of growth for humans in the pre-pubescent stage [189]. Indeed, in this study 9.3 cm was equivalent to 18 months of growth when calculated using the linear regression line for height and age in this cohort (linear equation, height [cm]=77 + (6.8 x Age [years]); not shown).
AMH levels in the boys did not correlate with the levels of other hormones known to regulate growth. IGF-1 correlated strongly with height and relative height, indicating that the GH-IGF-1 axis is an important predictor of growth. The strength of AMH as a predictor of a boy’s height was not degraded by the inclusion of IGF-1 in the linear regression models. This suggests that a boy’s level of AMH is a predictor of a boy’s height in addition to IGF-1 level. When IGF-1 level, age and parental height were accounted for, variation in AMH explained 9% of the variation in the height of the children. As a determinant of a boy’s current height, AMH levels explained as much variation as mid-parental height, a variable that documents most of the heritability of human height [240].

4.4.2 AMH and InhB may have redundant actions on growth

AMH is a novel predictor of height maturity in five- and six-year-old boys. However, the association of AMH with height could not be separated from the fellow Sertoli cell product, InhB. AMH and InhB correlated with the height of boys and finger length to a similar extent, even though the levels of AMH and InhB were only partially correlated. This suggests that InhB and AMH may be redundant regulators of growth, particularly as the sum of a boy's levels of InhB and AMH correlated more strongly with his height that either hormone alone.

Although AMH and InhB are both members of the TGF-β superfamily, their identified roles are independent and these proteins are not known to interact or share a signalling pathway. InhB is a feedback regulator of the hypothalamo-pituitary-testicular axis that controls spermatogenesis in men (like AMH, there are no known roles for InhB in the blood of boys) [246, 247]. However, mice that are deficient in both AMH and the inhibins develop testicular cancer more rapidly than mice deficient in either one of these hormones alone [248]. This indicates that AMH and InhB are convergent regulators of some cell types, although the mechanism underlying this convergence is currently unknown.
If the observed relationship between Sertoli cell hormones and height is causal, then the average boy in the study would be 5.2 cm taller on his 5th birthday, if he lacked InhB and MIS. This would create a boy-girl difference in height of 5% which is only slightly less than the 10% sex difference in men and women [189]. This suggests boys are the same height as girls at this age because the maturation of boys is retarded by the expression of AMH and InhB, which are both expressed at much higher levels in boys than girls at this age [249].

There may be evolutionary pressures to retard the growth of males during infancy and pre-pubescence. Firstly, if males matured at the same rate as females they would be bigger than females because of their larger adult size. During infancy, large males would require more nutrition from their parents to sustain their growth and in mammals that carry their young, males would require more effort to carry. During the pre-pubescent stage, fast growing males might struggle to meet their increased nutritional needs while learning to feed themselves and when competing for food. In this context, it is worth noting that AMH levels decline in boys with a time course that parallels the increased capacity of children to gather food in contemporary hunter-gatherer societies [19, 250, 251]. Thus, the males more likely to survive and pass on their genes could have been those that grew at a similar rate to females (and had similar nutritional needs) and spent a longer time in the pre-pubescent stage.

A correlation does not prove causality. Therefore, it is also possible that maturation in boys affects their Sertoli cell hormones. Sertoli cells express AMH at a very high level when they are in their immature state and at lower levels when they mature into their adult form at puberty [252]. However, the boys were five and six-years-old which is too young for normal pubertal development and thyroid hormone (T3), which is a hormonal regulator of Sertoli cell maturation, did not associate with AMH levels [253-255]. This indicates that AMH levels are not being affected by Sertoli cell maturation in five and six-year-old boys. Additionally, InhB levels increase with Sertoli cell maturation (Figure 4.23) so a negative correlation between InhB and height in boys cannot be explained by the maturation of their testes [252, 256].
The mechanism by which AMH or InhB may retard the growth of boys cannot be identified by correlative analysis. However, the presence of the AMH receptor, AMHRII, in the growth plate chondrocytes of fetal mice indicates that AMH may be a hormonal regulator of the growth plate. If AMHRII is also present in the human growth plate AMH may be inhibiting the expansion of the growth plate in pre-pubertal boys. It is therefore possible that the high levels of AMH and InhB seen in male infancy and prepubescence retard longitudinal bone growth.

It is, however, impossible to determine from the boy data what the independent effects of AMH and InhB on bone may be. The correlation between maturation and the Sertoli cell hormones raises three physiological scenarios; 1) that AMH inhibits development, 2) that InhB inhibits development, 3) that both InhB and AMH inhibit development redundantly.

### 4.4.3 Mouse growth

To determine the direct effects of AMH on growth, AMH null mutant mice were studied. If male AMH\(^{−/−}\) mice are the same as boys with low AMH, then it would be expected that AMH\(^{−/−}\) mice would be larger as pups and mature faster than wildtype mice. In contrast to this expectation, there was no significant difference in the length of bones in 20-day-old AMH\(^{−/−}\) mice when compared to their AMH\(^{+/+}\) littermates, indicating that AMH does not inhibit bone growth at this age. There was no significant difference in weight accrual or the timing of developmental milestones in AMH\(^{−/−}\) and AMH\(^{+/−}\) mice between the ages of 7 and 34 days old when compared to their wildtype littermates, suggesting that AMH may have no effect on the growth of male mice during the pre-pubescent stage.

However, adult AMH\(^{−/−}\) mice had significantly shorter bones than wildtype mice. This contrasts with the boy data which predicts that boys lacking AMH would mature faster than their peers,
but reach a similar adult height. Bone density was not affected in AMH−/− mice of either sex indicating that the effect is on bone length and thus likely to be localised in the epiphyseal plate. Therefore, at some point between 20 days and when the bone stopped growing (epiphyseal fusion around 70 days in mice) the bones of AMH−/− mice became shorter relative to AMH+/+ suggesting that AMH actually promotes longitudinal bone growth in mice. This time frame for the effect of AMH includes the transition into adolescence and into adulthood. The results of the longitudinal mouse growth study suggest that AMH may not be affecting the growth of mice before 34 days of age (adolescent stage). However, it is possible that skeletal differences may not be detectable by measuring weight in an animal as small as a mouse.

The effect of AMH on bone length in mice was the same in male and female mice. This is also consistent with AMH affecting bone growth during the adolescent period of growth. Little is known about AMH levels in female mice, but if their expression pattern matches other mammals (as it does in male mice, Figure 4.23) then female mice would not have significant AMH levels until adolescence.

The correlative data suggests that AMH may inhibit maturation of boys. If AMH has this role in mice, then it is possible that AMH may delay epiphyseal closure and thus lengthen the period of longitudinal bone growth. If this is the case, then AMH−/− mice of both sexes would have shorter bones because they undergo epiphyseal fusion earlier than wildtypes. Estradiol is a key regulator of bone maturation [216]. The actions of estradiol are postulated to by bi-phasic, that is, low levels of estradiol promote bone growth, whereas high levels of estradiol cause epiphyseal closure and thus prevent further bone growth [215]. Estradiol is a steroid hormone produced by aromatisation of androstenedione and androgens such as testosterone, this can occur in the gonads or within the target tissue [257]. AMH has been shown to inhibit aromatase activity in the ovary [55, 56]. Therefore, it is possible that AMH opposes the creation of estradiol in the epiphyseal plate and consequently delays its closure. However, it is also be possible that
AMH−/− mice have increased estradiol levels because of unopposed estradiol activity in the gonads.

AMH shares signalling machinery with the bone morphogenic proteins (BMP) (see general introduction) which makes it mechanistically possible for AMH to interact in BMP signalling, although no interactions have been reported. BMP signalling increases the growth of the epiphyseal plate [258-260]. Therefore, it is possible that AMH can oppose the growth of the epiphyseal plate by inhibiting BMP signalling.

4.4.4 Are the actions of AMH the same in mice and humans?

The human data suggests that AMH retards bone growth in pre-pubertal boys causing males to mature slower than females. Whereas, the mouse data suggests that AMH may delay the onset of epiphyseal closure in both males and females. There are five possible explanations for this data.

Firstly, it is possible that AMH does not inhibit the growth of the epiphyseal plate. Perhaps InhB regulates epiphyseal growth during pre-pubertal growth and AMH regulates a more subtle process within the bone at this time. This would explain the combined predictive power of AMH and InhB in a linear model and the unremarkable pre-pubertal development of the AMH+/− mouse. It is also possible that InhB is an inhibitor of pre-pubertal bone growth and AMH is an inhibitor of adolescent bone growth. In five and six-year-old boys there may be some overlap in these processes leading to an association between maturation and both these hormones.

Secondly, AMH and InhB may be redundant regulators of pre-pubertal growth. The effect of AMH deficiency in the AMH−/− mouse may be subtle because InhB can act as a substitute for AMH signalling in the growth plate. The AMH+/− InhB+/− combined mutant mouse develops tumours and cancer associated weight loss (cachexia) during pre-pubertal development [94] so it would be
difficult to determine if they mature faster than wildtype mice in the face of such a significant confounder.

The third possibility is that AMH has different effects on growth in humans and mice. Humans have a unique growth strategy with a prolonged pre-pubescent growth stage and the coincidence of sexual maturation and epiphyseal fusion creates a concise adolescent period compared to other mammals. Therefore, it is possible that AMH may delay the maturation of pre-pubertal bone growth in human males and not have any effect on pre-pubertal bone growth in mice. Consistent with this, male and female mouse pups are the same weight until 23 days old when males begin to grow heavier (laboratory records, not shown). However, AMH+/− mice weighed during this period were indiscernible from their wildtype littermates in weight indicating that AMH was neither maintaining males at the same size as females nor responsible for the male specific growth spurt. Alternatively, comparing AMH+/− mice to boys with low AMH may be inappropriate. Low serum AMH levels may mean that there is limited AMH signalling in target tissues, whereas a complete lack of AMH in any tissue may disrupt many systems (such as gonadal estradiol production or brain development). People with null mutations in the AMH gene are rare and poorly characterised in the literature. Therefore, it is unknown if AMH−/− humans have shorter bones similar to AMH+/− mice.

Finally, it is possible that AMH affects the maturation of bone growth during the both the pre-pubertal and adolescent stages of growth in both mice and humans. AMH is very high in males and at trace levels in females during the infant and prepubescent stages. High levels of AMH may retard the maturation of bone, so that males are less mature than females of the same age and coincidentally males are the same size as females during this time. It may be difficult to determine if the bones of male AMH+/− mice are maturing faster than normal (akin to female maturation) because the difference in skeletal size between male and female is much more subtle than humans. For instance, women have 11% shorter tibia than men on average (width 14%), whereas this study has shown the tibia of female mice is only 3% shorter (width 17%)
than those of male mice in within an inbred colony [261]. At puberty, AMH levels decrease in males and increase in females so that, by the end of adolescence, AMH levels are similar in males and females. Adolescent concentrations of AMH may regulate the production of estradiol and thus be a negative regulator of epiphyseal closure in both sexes.

4.5 Recommendations for further research

The research presented above shows an inverse correlation between AMH levels and the proportion of his expected adult height a boy has achieved at five or six years old. This suggests that the high AMH levels seen in normal boys may retard their maturation so that they are the same size as girls during childhood. However, this study does not determine if the AMH levels of girls also associates with height or proportion of expected adult height. AMH levels are much lower in young girls, but this does not rule out the possibility that low levels of AMH could be interacting with the growth plate in girls. The sample size of girls recruited in this study was too small for this analysis. Should a larger group of young girls be recruited, this analysis could help determine the nature of AMH signalling during childhood.

The boys in this study are normal developing boys. It would be interesting to study AMH levels in boys with pathologically altered rates of maturation such as those with precocious or delayed puberty. Additionally, further information could be gathered from studying boys with unusually high levels of AMH. For example, boys born to mothers with poly-cystic ovary syndrome have higher AMH levels, but not higher InhB levels, during infancy and childhood [262]. Whether these boys have an altered maturation rate or delayed onset of puberty has not been investigated.

Furthermore, it would be very informative to continue to measure AMH levels in the health boy cohort as these boys age. This longitudinal study would determine if the boys with high AMH
levels are in fact maturing slower than their peers. It would also be interesting to see if boys with high AMH levels at age 5 and 6 years continue to have high AMH levels throughout their development and into adulthood. Perhaps, the men with high AMH levels in Chapter 3 had high AMH levels during their childhood?

Analysis of the AMHRII reporter mouse and the AMH+/− mouse suggests that the association between AMH and development in boys may be due to a direct effect on the chondrocytes of the bone growth plate. In order to determine what effect AMH has on chondrocytes, primary cell cultures of growth plate chondrocytes could be treated with AMH. Chondrocytes could be observed for effects of differentiation and proliferation.

Additionally, the AMH+/− mouse strain could be studied further. These studies could involve scanning the bones of the mice at a greater variety of ages to include ages that are closer to epiphyseal closure (around 70 days old) or at the onset of puberty (around 15 days old). It could also be informative to look at ages where AMH levels are at their maximum (i.e. before 10 days in males, Figure 4.23) as this might when AMH signalling in bone is at its most active. It would also be informative to follow the bone growth of individual mice over a number of days. This approach is impractical at the moment, as the bone scanning equipment for mice requires that the mouse be euthanized or anesthetised. The latter may affect the mouse’s growth over the study period. The former would definitely have that effect.

The bones of the AMH+/− and AMH+/+ mice could also be examined histologically. The bones of mice from various ages could be decalcified and sectioned. Then the characteristics of the chondrocytes (size, shape, number) in each of the layers of the growth plate could be measured to determine which areas of the growth plate is affected by AMH signalling and what affect AMH is having on these chondrocytes. Markers of puberty such as kisspeptin positive neurons in the brain [263] could also be studied in the AMH+/− mouse to determine if AMH affects the maturation of other tissues beyond the growth plate.
Another approach to study the effect of AMH on development would be to give wildtype animals large doses of recombinant AMH at various stages of development to determine the effects AMH has on their growth and bone development. It may also be relevant to include doses of Inhibin in this regime in case the two hormones have a synergistic role. If this was performed in a slightly larger animal that could tolerate repeated blood sampling then hormones such as IGF-1 and T3 could be measured to confirm that AMH signalling is independent of these hormone levels.
Chapter 5: Regulation of AMH Expression

5.1 Introduction

Changes in hormone concentration signal to target cells information about the environment. These hormonal signals may convey short term information such as the amount of glucose available to cells (e.g. insulin [264]), body water content (e.g. vasopressin [265]), or whether it is day or night (e.g. melatonin [266]). Alternatively, information about long term states may be signalled such as starvation (e.g. thyroid hormone [267]), puberty (e.g. kisspeptin [268]) or pregnancy (e.g. chorionic gonadotropin [269]).

In order to signal this information effectively to target cells, the level of hormone in the blood must accurately reflect the state that it is signalling at the time (i.e. low insulin levels signals that glucose availability is low, high melatonin levels indicate that it is night time). Therefore, hormone concentrations must be controlled to effectively convey information.

If AMH is a hormone, then its serum levels must also be regulated. AMH levels are highly variable amongst individuals of the same age and sex indicating that AMH expression may have environmental determinants [17, 18]. However, no environmental factors have been shown to influence serum AMH, and factors such as weight loss [270], cigarette smoking [271], pregnancy [272] and hormonal contraceptive use [273] do not correlate with serum AMH levels. Unlike other hormones produced in the ovaries, AMH levels do not change during the different stages of the ovarian cycle (Section 1.4, page 18).

Despite the very different AMH levels between people of different ages and sex, little is known about regulation of AMH expression in general. Some clues about the regulation of AMH lie in the structure of the AMH gene.
5.1.1 Transcriptional regulation of AMH expression

Like all proteins, the production of AMH is controlled by the regulation of its gene. Transcription factors bind to dedicated sequences in the promoter region of genes and dictate how actively the gene is transcribed. Transcription is the first step in protein synthesis and is therefore a primary control point for protein production.

The AMH gene promoter contains conserved binding sites for three transcription factors known to be important for the regulation of AMH expression; SOX9, SF-1 and the GATA family of transcription factors [274-277].

SOX9 (SRY-related HMG box 9) is a transcription factor that is crucial for male sexual development [278]. When the early embryo is in a sexually ambiguous state, SOX9 is expressed at low levels in both males and females. SOX9 is then expressed at high levels in the developing testes after SRY gene activation, which is the first step in the pathway to male specific development [279]. SOX9 appears to be the main target for SRY because female mice overexpressing SOX9 in utero develop as males [278]. In humans, SOX9 deficiency leads to campomelic dysplasia, a generally lethal syndrome characterised by male to female sex reversal, bowing of the long bones and respiratory distress [280, 281]. The latter symptoms of campomelic dysplasia appear because SOX9 is also a key transcription factor for cartilage formation [282].

SF-1 (Steroidogenic factor 1) is another transcription factor crucial for AMH expression although its role is more complex than SOX9 [283]. SF-1 is expressed in developing adrenal glands and testes and is a nuclear receptor transcription factor meaning that its DNA binding is mediated by a ligand. Although this ligand has not been identified, the ligand binding domain appears to be important for Sertoli cell specific AMH expression [276, 283, 284].

SF-1 on its own is a relatively weak activator of AMH expression, however the SF-1 ligand binding domain can bind SOX9 to enhance transcription, possibly by altering the tertiary
structure of the AMH gene [274]. DNA binding domain of SF-1 can also interact with WT-1 (Wilms tumour protein 1) to increase AMH expression while binding of repressor protein, DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1), can negate the effects of WT-1 [285].

The role of SF-1 in AMH expression is further complicated by the presence of two conserved SF-1 binding sites within the AMH promoter meaning that two SF-1 molecules can bind the promoter in separate places and possibly recruit different ligands [276].

The third set of conserved DNA sequences in the AMH promoter bind the GATA family of transcription factors. Humans and other animals have two conserved GATA binding sites while mice and rat have only one [276]. Any member of the six strong GATA family should theoretically be able to bind the GATA sites, only GATA1 and GATA4 have been implicated in AMH expression regulation thus far [276, 286, 287]. GATA4 is expressed in prenatal Sertoli cells and upregulates AMH expression [286]. The DNA binding domain of SF-1 is also able to bind GATA4 to cooperatively increase AMH transcription [288]. DAX-1 is able to prevent this partnership to repress AMH expression [289].

In contrast to GATA4, GATA1 is expressed postnatally in the Sertoli cells [286]. GATA1 is also a repressor of AMH transcription and may be responsible for the pubertal decrease in AMH expression in males [287].

### 5.1.2 Vitamin D response element

Recently a fourth binding site has been found further upstream in the AMH promoter. A vitamin D response element (VDRE) is located -395 to -381 in the human AMH gene [2]. The vitamin D receptor binds the VDRE when bound to its ligand, vitamin D. In vitro studies demonstrate that
vitamin D is able to increase the transcription of AMH in prostate cancer cells and that this effect is also enhanced by the presence of SF-1 [2].

The vitamin D receptor is expressed by both granulosa and Sertoli cells raising the possibility that vitamin D could influence expression of AMH in these cells and thus the serum AMH concentration [290, 291]. This chapter will test whether circulating AMH levels are influenced by vitamin D.

### 5.1.3 Vitamin D

Vitamin D is a family of fat soluble compounds essential for calcium homeostasis and bone growth [292]. While vitamin D can be obtained through dietary means like other vitamins, very few foods are naturally high in vitamin D. The chief source of vitamin D in the body is biosynthesised in the skin after exposure to ultraviolet irradiation from sunlight [292].

Vitamin D deficiency is highly prevalent, especially during winter when the intensity of the sun decreases. Other factors such as dark skin pigmentation and cultural skin covering can decrease the amount of sunlight the skin absorbs and increase the risk of vitamin D deficiency.
There are two main input forms of vitamin D, vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Vitamin D2 is found in food derived from plants, yeast and fungi; and vitamin D3 is synthesised in the skin of humans and animals. As a dietary source, vitamin D3 has a higher bioavailability [293]. The liver converts vitamin D2 and D3 into calcidiol (25-hydroxyvitamin D3), the storage form of vitamin D, which is further processed by the kidneys to its active form, calcitriol (1,25-dihydroxyvitamin D3). Calcitriol has hormonal functions throughout the body [294]. Production of calcitriol is tightly managed and circulating vitamin D bound to vitamin D binding protein to control circulating concentration [295, 296]. Calcidiol levels are the best measure of a person's vitamin D status [297]. This chapter will therefore investigate the relationship between serum AMH and serum calcidiol levels in cross sectional studies of the human population.

5.2 Results

5.2.1 AMH levels correlated with vitamin D status in men

Calcidiol, the storage form of vitamin D, was measured in 113 of the healthy controls from the cardiovascular cohort. The mean serum calcidiol concentration was 111 nmol/L (range 2-317 nmol/L). Nine men had calcidiol levels below 25 nmol/L indicating vitamin D deficiency. Serum AMH concentration ranged from 1 to 87 pmol/L with a mean of 23 pmol/L. The level of AMH in the serum of the men positively correlated with their levels of calcidiol ($r=0.22$, $n=113$, $p=0.02$) (Figure 5.38). If vitamin D is a causal regulator of AMH levels, then the regression equation indicates that moving from vitamin D deficiency to the upper range of vitamin D levels would increase AMH levels by 13 pmol/L on average.

The association between calcidiol and AMH could reflect specific regulation of the AMH gene or the general effect of vitamin D on the metabolic activity of the Sertoli cells. If the latter, then
other Sertoli cell secretions, such as Inhibin B (InhB), should also co-vary with calcidiol. The levels of AMH and InhB were positively associated as expected ($r=0.40, n=113, p=0.000$). However, there was no association between InhB and calcidiol ($r=-0.01, n=113, p=0.92$). Furthermore, the inclusion of both InhB and age in a regression model did not affect the relationship between AMH levels and calcidiol (Table 5.15). This suggests that calcidiol correlates specifically with AMH levels rather than with the number or general function of Sertoli cells.
Figure 5.38. AMH levels correlated with calcidiol concentration in men.
Serum AMH and calcidiol were measured by ELISA in 113 healthy men aged 54-93 years. Men donated their blood at varying times of the year with more donating in winter than summer. AMH positively correlated with calcidiol by linear regression ($AMH = 0.04$calcidiol + 18.7, $r=0.22$, n=113, $p=0.021$).

Table 5.15. Calcidiol covaries with AMH independent of InhB and age in 113 mature men

<table>
<thead>
<tr>
<th>Model #</th>
<th>$r$</th>
<th>$p$</th>
<th>Predictor of AMH</th>
<th>$r$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.22</td>
<td>0.021</td>
<td>Calcidiol</td>
<td>0.22</td>
<td>0.021</td>
</tr>
<tr>
<td>2</td>
<td>0.50</td>
<td>&lt;0.001</td>
<td>Calcidiol</td>
<td>0.24</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>InhB</td>
<td>0.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>0.61</td>
<td>&lt;0.001</td>
<td>Calcidiol</td>
<td>0.22</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>InhB</td>
<td>0.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Age</td>
<td>-0.15</td>
<td>0.106</td>
</tr>
</tbody>
</table>

The relationship between serum AMH, calcidiol, InhB and age was examined using linear regression models; the partial correlates of the predictors in the model are also listed.
5.2.2 Vitamin D supplementation prevented the winter related decline of AMH in women

Vitamin D levels are influenced by sunlight exposure and are naturally lower during winter in people not taking vitamin D supplements [298, 299]. If vitamin D regulates AMH, then AMH should also be seasonal, with any seasonality being prevented by winter supplementation with vitamin D. This was examined using blood collected from a vitamin D intervention study, which compared summer and winter blood samples in a cohort of Dunedin women. Following the baseline summer blood test, the women were given either daily oral supplements of vitamin D or a placebo. Women receiving the placebo had a significant decrease in calcidiol levels during winter (average Δcalcidiol = -51 nmol/L, paired t-test \( p=0.001 \)). These women also had a significant seasonal effect in AMH with their winter levels being 18% lower on average their summer levels (average ΔAMH = -5 pmol/L, paired t-test \( p=0.01 \)).

In contrast, the women receiving 1000 IU of vitamin D3 supplements exhibited no seasonal loss of either calcidiol or AMH (average Δcalcidiol = +5.0 nmol/L, average ΔAMH = -0.3 pmol/L, paired t-test \( p=0.05 \) and \( p=0.83 \), respectively; Figure 5.39). Supplementation with the less potent D2 (1000 IU daily) did not prevent the winter decline in either calcidiol or AMH (average Δcalcidiol = -25 nmol/L, average ΔAMH = -2.7 pmol/L, paired t-test \( p=0.003 \) and \( p=0.03 \), respectively, Figure 5.39).

If vitamin D directly regulates AMH then the magnitude of a woman’s ΔAMH should correlate with the magnitude of her Δcalcidiol. This was examined by combining the three treatment groups (placebo, D2, D3) to generate a single cohort of women with a wide range of Δcalcidiol, which was then examined using linear regression. The ΔAMH of the women correlated with Δcalcidiol and their initial AMH concentration (Figure 5.40). The initial level of AMH is related to age, as the number of follicular cells producing AMH declines as ovarian reserve decreases [300, 301]. The inclusion of age and the initial calcidiol concentration did not significantly alter
the partial correlates between Δcalcidiol and ΔAMH, indicating that these variables do not profoundly affect the vitamin D regulation of AMH. Specific studies would, however, be needed to exclude subtle effects of ageing or ovarian disease on the regulation of AMH expression.

5.2.3 **AMH did not correlate with vitamin D status in boys**

Boys have much higher serum AMH concentrations than adults (Section 1.4, page 18). This indicates that AMH expression in boys may be driven by powerful transcription factors not present in adults. Therefore, calcidiol was measured in 74 boys from the five- and six-year-old cohort to determine if the AMH promoter is sensitive to vitamin D when strongly activated. Participants donated their blood at varying times of the year which resulted in a wider range of calcidiol concentrations (3-237 nmol/L, average 78 nmol/L). Ten boys exhibited calcidiol levels indicative of deficiency. There was no correlation between AMH and calcidiol in the boys \( (r=0.07, p=0.54, \text{Figure 5.41}) \), suggesting that vitamin D does not regulate AMH in boyhood. Inclusion of InhB in a linear regression model to control for Sertoli cell number did not change the relationship between calcidiol levels and AMH \( (r=0.07, p=0.62) \).
Figure 5.39. AMH and calcidiol levels were seasonal.
Women were given daily doses of vitamin D3 (n=16), D2 (n=7) or a placebo (n=10) over autumn and winter months. The winter levels of calcidiol (blue) and AMH (yellow) are illustrated as the percentage of each woman’s summer levels. The bar represents the mean + the standard error of the mean. # illustrates significantly different to the summer values when tested with paired student’s t-test #1 $p=0.001$, #2 $p=0.01$, #3 $p=0.003$, #4 $p=0.03$.

Table 5.16. Changes in AMH levels correlated with seasonal changes in calcidiol levels in 33 women

<table>
<thead>
<tr>
<th>Model</th>
<th>$r$</th>
<th>$p$</th>
<th>Predictor of $\Delta$AMH</th>
<th>$r$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0.002</td>
<td>$\Delta$Calcidiol</td>
<td>0.38</td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial AMH level</td>
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<td>0.002</td>
</tr>
<tr>
<td>2</td>
<td>0.62</td>
<td>0.007</td>
<td>$\Delta$Calcidiol</td>
<td>0.45</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial AMH level</td>
<td>-0.52</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial calcidiol level</td>
<td>0.21</td>
<td>0.271</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Age</td>
<td>0.20</td>
<td>0.297</td>
</tr>
</tbody>
</table>

The relationship between the change in a woman’s level of AMH ($\Delta$AMH) and the change in her level of calcidiol ($\Delta$calcidiol) were analysed using linear regression models, with the influence of the initial levels of AMH and calcidiol being studied. The partial correlates from each model are recorded, and the partial plot of model 2 illustrated in Figure 5.40.
Figure 5.40. Partial plot of linear regression presented in Table 5.16. The association between ΔAMH and Δcalcidiol is shown adjusted for the initial levels of AMH and calcidiol ($r=0.36$, $p=0.004$). Regression equation: $\Delta$AMH = 0.08(Δcalcidiol) - 0.16(initial AMH) + 0.043 (initial calcidiol) - 1.15.

Figure 5.41. The levels of AMH did not correlate with calcidiol levels in boys. Calcidiol was variable (3-237nM) in 74 boys aged 5-6 years sampled at varying times of the year, but there was no correlation between AMH and calcidiol in a linear regression (AMH = 0.001(calcidiol) + 0.89, $r=0.07$, n=74, $p=0.541$).
5.2.4 The vitamin D response element is not conserved across species

Serum AMH correlates with calcidiol levels in adults which is consistent with vitamin D regulating the transcription of the AMH gene via the vitamin D response element (VDRE) in the AMH gene promoter. AMH is regulated by several transcription factors important for early embryonic development. The binding sites for these factors are highly conserved amongst mammals [276]. To determine if the vitamin D receptor binding site, VDRE, is similarly conserved, the AMH promoter regions were compared in five mammalian species (pig, cow, human, mouse and rat). At least 300bp upstream of the transcription start site was included in a multiple sequence alignment for each species. Conserved elements such as SF1, SOX9 and the GATA sites were obvious in the aligned sequences (Figure 5.42). Further upstream from the other transcription factor sites, the VDRE sequence was identified in the human sequence. This motif was degraded in the other species, with the cow sequence having the most base pairs of the motif (7/17). The VDRE consensus sequence (GGGTCANNGGCA) is based on ten base pairs so it is possible the VDRE motif may bind the vitamin D sequence in the cow [1]. The pig, mouse and rat sequences had only had three base pairs in common with the human motif suggesting that the VDRE is not functional in these species.

To determine if the VDRE element is present in species closely related to humans, AMH promoter sequences for primates (orangutan, gibbon, marmoset and chimpanzee) were aligned. Conserved binding sites, SOX9, SF1 and GATA were conserved between these species (not shown). The chimpanzee and human sequences were identical in the 300bp studied. Thus, the chimpanzee had the identical VDRE binding site found in humans (Figure 5.43). There was a 12bp insertion in the VDRE element in the orangutan, gibbon and marmoset sequences, indicating that the VDRE element in these species is unlikely to be functional despite conservation of most of the bases in the VRDE (orangutans and gibbons 10/17, marmoset 12/17).
The vitamin D receptor is known to bind many varied motifs [302], meaning that vitamin D may regulate AMH expression in other animals independent of the human VDRE identified. Alternatively, a VDRE element may be present in other mammalian species, but the motif location may not be conserved. To determine if vitamin D can alter AMH gene expression, male mice were given one large oral dose (2μg/kg) of calcitriol, the active form of vitamin D, and their testes were removed for real time-PCR analysis three days later. On average, the amount of AMH mRNA produced in the testes of mice treated with calcitriol was 32% higher than mice treated with the sesame oil control (Figure 5.44). However, this did not reach statistical significant indicating that the study may be underpowered. Therefore, it remains unknown if mice have a functional VDRE in their AMH gene.
Conserved regions in AMH gene promoter.

AMH promoter sequences from pig (S. scrofa gi34761.8792), cow (B. taurus gi35547.186), human (H. sapiens gi224589810), mouse (M. musculus gi372099100) and rat (R. norvegicus gi34761.8792) species were retrieved from the NCBI gene database and aligned with Clustal W2 multiple sequence alignment tool (EMBL-EBI) using default settings. At least 300bp of the promoter sequence upstream of the transcription start site has been included for each species.

Conserved binding sites were then identified using information from the literature and unidentified conserved regions were given putative annotation using the CISRED database (cisred.org) [2, 276]. Conserved base pairs (bp) have been marked in bold. VDRE = vitamin D response element, SF1 = Steroidogenic factor 1, p53? = putative p53 binding site, NRSF? = putative neuron restrictive silencer factor binding site.
Figure 5.43. Alignment of the VDRE element in the AMH promoter in primates.
Entire AMH promoter sequences for all primate species present in the NCBI database, human (H. sapiens gi224589810), chimpanzee (P. troglodytes gi291061357), orangutan (P. pygmaeus gi241864923), gibbon (N. leucogenys gi328833343) and marmoset (C. jacchus gi290467387), were retrieved and aligned with the Clustal W2 multiple sequence alignment tool (EMBL-EBI) using default settings. At least 300bp of the promoter sequence upstream of the transcription start site has been included for each species, only the alignment of the VDRE region is shown for clarity. VDRE consensus sequence was taken from the literature and aligned by hand [1]. Bases have been highlighted if they match the VDRE identified in the human gene [2]. Conserved base pairs have been shown in bold.

<table>
<thead>
<tr>
<th>Species</th>
<th>AMH Promoter Sequence</th>
<th>VDRE Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>orangutan</td>
<td>GGATCTGCAGAGGAGACCAAGGAGA</td>
<td>GGGTCA</td>
</tr>
<tr>
<td>gibbon</td>
<td>GGGTCTGCAGCGCCGGATCACAAGGAGA</td>
<td>NN-GGG-CA</td>
</tr>
<tr>
<td>marmoset</td>
<td>GGGTCTGCAGCGCCGGATCACAAGGAGA</td>
<td>SCAGGGGAGGGCCCCCCC</td>
</tr>
<tr>
<td>human</td>
<td>GGGTCTGCAGCGCCGGATCACAAGGAGA</td>
<td>SCAGGGGAGGGCCCCCCC</td>
</tr>
<tr>
<td>chimps</td>
<td>GGGTCTGCAGCGCCGGATCACAAGGAGA</td>
<td>SCAGGGGAGGGCCCCCCC</td>
</tr>
</tbody>
</table>

Figure 5.44. Vitamin D may not regulate AMH expression in mice.
AMH mRNA expression was measured by real time PCR in mice dosed with vitamin D (yellow, n=8) and mice given a sesame oil control (blue, n=10). There was no significant different in AMH mRNA expression between the treated and control groups (Student’s t-test p=0.13). Mice were either 25 days old or 80 days old, the different age groups were normalised to the mean of their controls before being combined. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control gene.
5.2.5 Other conserved areas in the AMH gene promoter

In addition to known transcription regions and the VDRE, there were three more conserved regions within the divergent species. There was a strongly conserved CAGGCC motif located between the distal GATA and distal SF-1 sites (Figure 5.42). When compared to a transcription factor binding site database (CISRED database) this motif was identified as a putative p53 binding site. The other conserved region, AGGCG(AG/CA)CCC(A/G)G, was a putative neuron restrictive silencer factor (NRSF) binding site located between the TATA box and the transcription start site. The third region, G(A/G)ACAGAA, did not match any transcription factor binding sites in the database.

5.3 Discussion

AMH levels vary considerably between individuals of the same age and sex [17, 18]. Several lines of evidence suggest that vitamin D status is one of the factors responsible for this variation in men and women. First, the promoter of the AMH gene contains a vitamin D response element which is active in cultured cells [2, 7]. Second, the serum concentration of AMH in men correlated with serum calcidiol levels. This is consistent with the direct regulation of AMH by vitamin D, as the levels of the other major Sertoli cell hormone, InhB, did not correlate with calcidiol. Third, women experienced a winter decline in AMH levels which correlated with the change in their calcidiol concentration and effective vitamin D supplementation during winter prevented AMH levels from decreasing. Collectively, these observations implicate vitamin D as a determinant of circulating AMH concentration.

The average winter decrease in AMH values of women without vitamin D supplements was 18%. AMH also decreases in mature women as their ovarian reserve diminishes, with an 18% decrease in AMH being approximately equivalent to 2 years of aging closer to menopause [303].
Addition of age in the regression analysis did not diminish the relationship between Δcalcidiol and ΔAMH, indicating that any decrease in AMH due to decreased vitamin D in a woman is independent to any decline she may be experiencing from aging.

These results are the first evidence that an environmental factor (i.e. sunlight exposure or vitamin D intake) directly affects the amount of AMH in human blood. This is also evidence that AMH has hormonal functions because hormones require regulation of their concentration in the blood. Like AMH, vitamin D has a long half-life in the blood and is relatively steady over time [304, 305]. This indicates that AMH may be signalling target cells information about long term processes such as whether it is summer or winter. Subsequent studies will be needed to determine why AMH is regulated by vitamin D.

Vitamin D deficiency is highly prevalent, in the cross-sectional study 8% men were deficient and in the intervention study, 30% of the women were vitamin D deficient during winter. Some populations, such as those with dark skin pigmentation, will have much higher risk of vitamin D deficiency. This may lead to decreased serum AMH levels. Consistent with this, AMH varies with race in American women, with black women having lower AMH levels than white women of the same age [306].

As little is known about the hormonal functions of AMH, it is hard to predict what the symptoms of chronically low AMH levels would be. Would, for instance, a person with low vitamin D and low AMH be at an increased risk of developing an abdominal aortic aneurysm (see Chapter 3)? It is interesting to note that Vitamin D and AMH share some similarities as both have been implicated in the regulation of gonadal aromatase activity and sperm viability [55, 82, 307]. Vitamin D has also been shown to alleviate some of the symptoms of polycystic ovary syndrome (PCOS), an endocrine disorder where many immature follicles develop in the ovaries[308]. Due to the high number of developing follicles serum AMH is much higher in PCOS women than the normal reference range [309]. At first glance, it makes little sense as to why increasing vitamin
D, and presumably further elevating AMH levels, would improve PCOS symptoms. However, it is possible that while the overall AMH output from the ovaries is high in PCOS women, the AMH output per follicle may be insufficient for signalling within its local environment (i.e. for repressing further follicles from maturing, see Section 1.6.5.1, page 28).

While further research will be required to determine the implications of altering an individual’s AMH level, there is already one clinical implication for the research findings in this chapter. In recent years, AMH levels have become a popular measure for gonadal status, particularly ovarian reserve in women [300]. Low maternal AMH is a predictor of poor success rate in assisted fertility procedures such as in vitro fertilisation [301]. The current study suggests that vitamin D status affects a person’s AMH concentration. Consequently, vitamin D deficiency may need to be considered when serum AMH levels are obtained for clinical diagnosis.

In contrast to men and women, there was no association between AMH and calcidiol levels in boys. AMH expression in boys is very high compared to adults. This suggests that there are boy-specific enhancers driving AMH transcription. This may mean that any influence of vitamin D on AMH expression is limited to a small enhancement of an already very active transcription site. Alternatively, it is also possible that the presence of childhood activators prevent the vitamin D receptor from binding the AMH promoter or from interacting with the SF-1 transcription factor.

Although parts of the VDRE were conserved in divergent species, in most species the VDRE was too degraded to be presumed functional. Humans and chimpanzees were the only species with an entire VDRE motif that was similar to the VDRE consensus site previously published. It is worth noting however, that the cow promoter contains the second half of the human VDRE and it may be possible that this motif can bind vitamin D.

Dosing mice with high doses of active vitamin D (calcitriol) yielded inconclusive results, so further studies would need to be completed to determine if mice have a functional VDRE element in their AMH gene. However, links between AMH and vitamin D have been reported.
Increased light exposure has been associated with increased serum AMH levels in the Siberian hamster [310]. Conversely, treating cultured chicken granulosa cells with vitamin D decreases AMH transcription [311]. The chicken AMH gene promoter has a different structure to the mammalian sequences. This indicates that the structure of the AMH gene may determine whether vitamin D is an activator or repressor of AMH transcription. These reports suggest that more species, beyond primates, have a VDRE in their AMH gene promoter. Unfortunately, the AMH gene sequence for the Siberian hamster was not available for analysis and the chicken sequence is too dissimilar to the mammalian species for alignment so these VDRE sequences remain unknown.

Putative binding sites for p53 and neuron restrictive silencer factor (NRSF) were also identified in the AMH gene promoter. Studies that target these elements directly will be needed to determine if these putative elements are functional. However, they raise the possibility that AMH expression is regulated by the p53 family or NRSF.

The p53 family consists of three members, p53, p63 and p73, all of which are important cell cycle regulators [312]. AMH may therefore be part of the cell cycle regulation pathway. Consistent with this, AMH has been shown to upregulate other cell cycle regulators, p50, p65, p16, p107 and p130 in cell culture [29-32, 87-90].

NRSF is a protein expressed throughout the body that prevents expression of neuronal genes in non-neuronal tissue [313]. This specific inhibition is accomplished by binding the NRSF element found in neuronal genes. The gonads are the chief source of AMH in circulation, however, AMH is also produced endogenously in mature neurons [6, 8-10]. The putative NRSF binding site found in the AMH gene is located between the TATA box, which is a general transcription factor site that recruits the RNA polymerase, and the transcription start site. If a protein were to bind the AMH gene at this location it would prevent gene transcription which is consistent with the role of the neuron restrictive factor in neuronal genes. Thus, NRSF may prevent AMH expression in...
most tissues. It is not known if NRSF is expressed in the Sertoli or granulosa cells of the gonads where AMH is highly expressed.

In conclusion, serum AMH appears to be regulated by vitamin D in men and women. Controlling for vitamin D status may increase the accuracy of AMH levels obtained for clinical diagnosis. These findings also provide further evidence that AMH has hormonal functions as hormones require their concentration in the bloodstream to be regulated.

5.4 Recommendations of further research

Human samples have been taken from various blood banks which were created for various purposes and therefore this study has some weaknesses that a cohort recruited specifically for an AMH and vitamin D study would not have by design. For example, no information is available about factors such as vitamin D supplementation or sunlight exposure in the adult male cohort and thus it is not possible to determine if there is a seasonal effect on AMH like that seen in women. Also, the intervention cohort (women) does not increase vitamin D concentration, so it is not possible to determine the exact effects of increasing vitamin D on AMH levels.

It would be nice to include an acute dosing trial in this thesis in which a cohort of healthy men and women deficient in vitamin D would donate a blood sample before and several time points after a single, large dose of vitamin D. This would help define the size and timing of the increase in AMH concentration in response to vitamin D. This information would be useful clinically (i.e. what is the best way to correct for vitamin D deficiency when taking an AMH sample for diagnosis?) as well as experimentally (i.e. increasing AMH levels in people at risk of blood vessel disease). This study would possibly extend to a cohort of fertility patients with known low AMH to determine if very low AMH levels are affected by vitamin D. These studies rely on recruiting a large cohort of healthy people deficient in vitamin D (to prevent vitamin D toxicity in
participants). Realistically, this study can only be done during the end of winter (July-August) and so could not be included in this thesis due to time constraints.

The comparison of AMH gene promoter sequences from different species in this chapter has identified two putative transcription factor binding sites for p53 and NRSF. Unlike the vitamin D response element, these transcription factor sites do not bind a receptor with hormonal ligands. Instead, p53 and NRSF are produced endogenously in the cell. This means that the activity of these putative regulatory sites cannot be investigated with the serum correlation approach used in this chapter. Instead, the best way to determine if these sites are active would be to selectively remove the base-pairs of the putative sites to determine the effect of AMH expression. This technique would involve creating a series of expression vectors that contain the AMHRII promoter (with its natural sequence and with either or both of the putative p53 and NRSF sites mutated) ligated to a reporter gene sequence (i.e. firefly luciferase or green fluorescent protein), these constructs would be transfected into a cultured cell line (preferably one with low NRSF and p53 activity, such as a neuronal cell line) and these cells would be treated with recombinant NRSF or p53. If the putative sites are active, then treatment with these proteins will induce a change in reporter gene activity in the cells transfected with the non-mutated promoter and not in those missing the respective transcription site. If NRSF or p53 are inhibitors of AMH transcription (as NRSF is expected to be) then a known activator of AMH expression (such as SF-1) may need to be used to activate the reporter gene before treatment with the putative inhibitor.

This technique could be expanded to test the interactions between the known transcription factors of AMH. Such a study may explain the complex patterns of AMH secretion in the blood and yield further information about which cell types are likely to express AMH.
Chapter 6: Final Conclusions

The aim of this thesis was to investigate if AMH is a hormone. At the beginning of this thesis it was concluded that AMH completely fulfilled two of the criteria of a hormone. That is, AMH is biosynthesised in a secreting cell and then secreted into the blood.

The criterion that AMH must be recognised by receptors in target tissue was met by a few reports of AMHRII expression throughout the body, although there were very few locations of AMHRII expression outside of the reproductive system.

Likewise, AMH appeared to meet the criterion that AMH levels in the blood must change to convey information because AMH levels change during development and are highly variable between individuals, however, it was not known circulating AMH concentration was controlled by any environmental factors.

Finally, AMH failed to meet one criterion entirely. Hormones must have a function, that is, hormones must stimulate a response of some kind from the target tissue. At the beginning of this thesis, there were no putative hormonal functions of AMH in the blood of children or adults.

This section will summarise the findings of this thesis in context to the two partially met and one failed criteria.

6.1 AMHRII appears to be expressed extensively throughout the body

The results of this thesis suggest that AMHRII expression may be extensive throughout the body and involve many tissues outside of the gonads and associated reproductive structures. Analysis of the AMHRII reporter mouse suggested that AMHRII expression may occur extensively
throughout the fetus, with staining in cells of the cardiovascular system, respiratory tract, gastrointestinal tract, central nervous system, visceral organs, skin, eye, tendons and skeleton.

The tissues of the adult reporter mouse were not as intensively studied due to the fact that the whole mouse could not be sectioned intact as can easily be done with a fetus. Nevertheless, staining was confirmed in the heart, blood vessels and cartilage in the adult AMHRII reporter mouse with other tissues having negative or inconclusive results.

The LacZ staining method used in this thesis is an indirect measure of AMHRII expression because LacZ expression is constitutive once activated. Therefore, the findings from this thesis require validation with approaches that detect the expression of AMHRII directly in mouse and, more importantly, human tissues.

6.2 Circulating AMH has putative functions

AMH levels correlated with biological measurements in both men and boys suggesting that circulating AMH associates with tissues in a manner that is consistent with endocrine actions.

In men, AMH levels inversely correlated with aortic diameter and AMH levels were significantly lower in men with aortic aneurysms. This indicates that AMH may be a hormonal regulator of blood vessel size. Consistent with this, the AMHRII reporter mouse indicated that AMHRII may be expressed in the tunica media of blood vessels. The presence of AMHRII in blood vessels needs to be confirmed in human samples before it can be concluded that AMH signalling is occurring in the blood vessel wall. If this is the case, then the cellular mechanism that AMH signalling induces in the medial layer of the vessel wall will need to be determined. This will require studying the effect of manipulating AMH levels on the structure of the blood vessel wall and other risk factors for vascular disease. Such a study cannot be performed in humans, so an animal model such as the AMH−/− mouse will be required.
In boys, AMH levels negatively correlated with current height and amount of expected adult height achieved, indicating that AMH may be a hormonal regulator of bone growth. However, height parameters also correlated with inhibin B which is another Sertoli cell product. The association between boy height measures and AMH was unable to be isolated from the association with inhibin B making it difficult to determine the effects of AMH alone. LacZ staining in the AMHRII reporter mouse indicated that AMHRII may be expressed in the bone growth plate indicating that AMH signalling may directly affect longitudinal bone growth. Consistent with this, the results of the AMH−/− mouse bone analysis indicated that AMH may have a direct action on bone growth with AMH−/− males and females maturing with significantly shorter bones than their wildtype littermates.

However, young AMH−/− mice did not have significantly different bone lengths or maturation rates than their wildtype littermates which conflicted with the boy data. This indicates that there is complexity in the AMH-bone growth relationship that requires further study. The first priority would be to determine what effect AMH (and possibly inhibin B) has, if any, on the proliferation and differentiation of the chondrocytes within the bone growth plate.

6.3 Circulating AMH is biologically regulated

Prior to this thesis, there were no environmental factors shown to influence circulating AMH concentration. The findings in this thesis show that AMH levels correlate with vitamin D levels in men and women (but not boys). A fall in AMH levels in women during winter was also observed and this winter decline in AMH concentration was abated when the women took vitamin D supplements. These findings indicate that a vitamin D response element discovered in the AMH gene by another research group [2] is active in vivo and that vitamin D is a regulator of circulating AMH concentration.
Gene sequence analysis indicated that this regulatory element may be unique to humans and close primate relatives. This analysis also identified two putative regulatory elements that have not been previously identified. These elements require further study to determine if they are functional. If they are confirmed to be transcription binding sites then this will provide further information about the regulation of AMH expression and give clues about what conditions and in which cells AMH is likely to be expressed. This may help identify further actions of AMH.

Regulators of AMH expression are also important confounders when analysing AMH levels. Therefore, analysis of AMH levels may be less accurate if the person being sampled is vitamin D deficient. In this study, vitamin D was not a confounder to the correlations investigated.

Vitamin D is unlikely to be the only environmental determinant of AMH levels, thus the exact effect of vitamin D intake on AMH levels cannot be calculated from the research in this thesis. Therefore, an acute dosing study that determines the timing and magnitude of the change in AMH levels after a dose of vitamin D is needed. This will allow for the effects of vitamin D regulation to be accurately considered in the clinic where AMH may be taken for diagnostic purposes or in scientific studies that may wish to alter AMH levels in human participants.

6.4 AMH is a putative hormone

The research findings outlined in this thesis show that AMH receptors may be expressed in many different tissues outside the gonads, also, that circulating AMH levels are regulated and that AMH levels correlate with blood vessel size in men and height in boys. This is novel evidence that AMH in the blood is not simply passive leakage from the gonads, but that AMH is being actively secreted into the blood to perform hormonal tasks.

However, further study is required to confirm that AMH has hormonal actions in the bone growth plate and blood vessel wall. So, for now, AMH is a putative hormone because it has
putative functions in the blood, which are not yet confirmed. AMH is much closer to being classified a hormone after this research but, at present does not completely fulfill the criteria required of a hormone.

AMH is a member of the TGF-β superfamily. Members of the TGF-β superfamily are typically involved in a variety of roles in development and homeostasis (Sections 1.6 and 3.1.4). The results of this thesis indicate that AMH is not an exception within this family rather that the lack of known AMH endocrine roles stems from a lack of investigation into AMH biology. In fact, if AMH is shown to have a direct effect on the growth or differentiation of chondrocytes in the bone growth plate, then AMH would classify not only as a hormone but also as a member of the bone morphogenetic protein (BMP) subfamily within the TGF-β superfamily.

6.5 Implications of AMH deficiency

In this thesis, there was a wide range of AMH levels within cohorts of similar people (i.e. grouped by sex and age) which is consistent with other reports [17, 18, 314]. However, the distribution of AMH levels has not been commented on in other reports. In this thesis, the distribution of AMH levels in the healthy men and boys was skewed toward higher levels despite the ranges of AMH levels being very different in these two cohorts.

This indicates that there may be a lower threshold for circulating AMH concentration and consequently lower AMH levels are underrepresented. An AMH threshold could occur because low AMH levels are inconsistent with survival and reproductive fitness, or because people with low AMH levels are excluded from study in some way. Males with no AMH expression, although very rare, do survive gestation and childhood indicating that AMH is not a crucial survival factor for males, however the presence of Müllerian ducts in AMH null men may hinder fertility and their ability to pass on their genes [41, 315, 316]. However, the fact that AMH null men can be
born does not mean that every fetus with persistent Müllerian duct syndrome survives in utero. The fetal AMHRII reporter mouse analysis (Chapter 2) suggests that AMH signalling may be widespread throughout the fetus. Therefore, it is possible that male fetuses with low or no AMH expression are more likely to die in utero. Pregnancy has a high failure rate (around 20% of confirmed pregnancies in humans [317]) so this mechanism is not unlikely. However, the ratio of AMH−/−, AMH+/− and AMH+/+ mice born from AMH+/− x AMH+/− matings match the Mendelian ratio for a heterozygote mating (1:2:1, laboratory records) indicating that AMH−/− are not at a greater risk of dying in utero. AMH−/+ mice also do not have a greater risk of dying postnatally (Mclennan, personal communication). That being stated, there is no data available on the distribution of AMH levels in mice to determine if mouse distribution is also skewed towards higher AMH levels.

The inclusion criteria for the human cohorts were quite liberal with study volunteers required only to be healthy and within a certain age range. It is possible that boys and men with low AMH levels were less likely to be recruited into the study because they did not meet health criteria. The boy cohort consisted of five- and six-year-old boys attending school, which is a population that generally do not have serious health problems or a high mortality rate (New Zealand child mortality rate for the year of this study was 6.2 per 1000 children, United Nations Statistics Division). Also, this study did not exclude volunteers with common childhood ailments such as asthma or allergies. This indicates that general study design probably did not exclude boys with low AMH levels unless, of course, these children were less likely to be attending school or less likely to agree to participate in this study.

In the healthy male cohort a selection bias in the distribution of AMH levels is more likely because medical testing was used to define the healthy cohort. There was a strong selection against cardiovascular disease in this cohort and men with abdominal aortic aneurysms (who have lower AMH levels, Chapter 3) were excluded. These selection criteria may explain the
shape of the distribution plot in the men, however AAA incidence is relatively rare compared to other cardiovascular events (AAA incidence 5.6 per 100,000 people, myocardial infarction 287 per 100,000 people [318, 319]), so the exclusion of AAA patients from the healthy cohort could be expected to have little effect on the distribution of the general population. It is possible that people with low AMH are also susceptible to other forms of cardiovascular disease, or disease in general, which have not been directly tested in this thesis. The majority of men were sampled in winter so this AMH threshold cannot be explained by a sunlight exposure (and thus vitamin D) keeping AMH production at a minimum level.

It is not known if AMH levels are stable throughout life. It could be that the men with lower AMH levels in this study were once boys with lower AMH levels. If this is the case, then the distribution of AMH levels in boys and men could be related and have the same underlying cause.

The women and girl cohorts were not large enough to analyse the distribution of AMH levels. However, AMH is also in the blood of in girls and premenopausal women for no known reason. At menopause, AMH levels become undetectable in women. If AMH has hormonal roles such as regulating blood vessel wall function or skeletal homeostasis, then there may be health implications that follow the loss of AMH expression at the menopause transition. For example, the incidence of cardiovascular disease increases in menopausal women [320]. Further studies are therefore needed to investigate the hormonal roles of AMH in premenopausal women and the effects of losing AMH expression at menopause. Therefore AMH levels require further study in women.

The apparent lower AMH threshold level seen in men and boys indicates that AMH deficiency may have implications for health and fitness. The results of this study indicate that these health implications may include vascular and skeletal health. This thesis presents the first ever study of
hormonal AMH and it is likely that further study will yield more putative hormonal roles and subsequent health implications of low AMH.

6.6 Limitations of research

The results presented in this thesis cover four novel studies of AMH biology. These studies cover a wide range of topics from the epithelium of the fetus, to vascular health, to child development, to control of AMH gene transcription. This means that, while the presented research into AMH hormonal function is broad, each individual study has parts of it that remain at preliminary stage. For example, there was no time to investigate the other putative AMH target tissues identified with LacZ staining in Chapter 2, or to test the activity of the putative regulatory sites in the AMH gene promoter in Chapter 4. Recommendations for further research in each of these studies are included in each research chapter.

In order to research AMH levels in so many different types of people and conditions, this study had to rely on the generous help of researchers who had blood samples left over from cohorts recruited for other studies. This means that some of the cohorts have not been constructed to control for variables that would be considered in an endocrine study, such stage of ovarian cycle, fertility issues or the time of the day the blood was taken (important for testosterone measurements). Not controlling for these variables probably had little bearing on the AMH correlates since AMH levels are remarkably stable, but it may have introduced some noise into the analysis.
Chapter 7: Methods

7.1 Human Studies

7.1.1 Men cohort

Serum from 153 healthy men (aged 54-93 years) and 69 men with abdominal aortic aneurysms (aged 54-88 years) was acquired from the Vascular Research Group blood bank run by Assoc. Prof Gregory Jones and Prof Andre van Rij. This blood bank also supplied plasma samples from 70 men with peripheral artery disease (aged 40-87 years) and 70 men with varicose veins (aged 22-90 years). Samples were transferred on dry ice and stored at -80°C.

The healthy cohort was comprised of study volunteers with no history of cardiovascular disease and an ankle-brachial pressure index between 0.7 and 1.5 upon clinical examination. The abdominal aortic aneurysm (AAA) group were men with a maximal infrarenal aortic diameter greater than 3cm on clinical examination or who had had an AAA rupture that required surgical repair. The varicose vein group were men that were diagnosed with chronic venous insufficiency this was confirmed by duplex venous scan and plethysmography. The peripheral artery group were men with a previous diagnosis; this was confirmed with resting ankle-brachial pressure index (less than 0.7), pulse volume recordings, and symptomatic stenosis identified by arteriography and/or duplex arterial scan. AAA, varicose vein and peripheral artery disease patients were recruited from patients referred for clinical assessment to the Otago Vascular Diagnostics laboratory and the Vascular Surgical outpatient’s clinic at the Dunedin Hospital.

In addition to the blood samples, the blood bank also shared clinical information collected about the participants. This included ultrasound measurements of: the external diameter of the abdominal aorta (4-7MHz) at three sites of the abdominal aorta; suprarenal (immediately distal to the superior mesenteric branch ostium), distal-infrarenal (1cm proximal to the aortic
bifurcation) and mid-infrarenal; and the intimal-medial thickness of the carotid artery measured 1cm proximal from the bifurcation in the common carotid artery (7-12MHz) in accordance with the Mannheim consensus statement [321].

As well as results from blood non-fasting blood tests which was analysed by the Vascular Research Group for low density lipoprotein, high density lipoprotein, cholesterol and triglyceride levels and high sensitivity C-reactive protein levels that were processed in the Otago Vascular Diagnostics laboratory. Information on age, body measurements, blood pressure, prescribed medicines, medical history and smoking history were also supplied.

The study was approved by the University of Otago Human Ethics Committee.

### 7.1.2 Child cohort

The blood from 102 boys and 30 girls was acquired from a behavioural study run the MIS neurobiology group in a collaboration with Prof David Bilkey and Prof Ted Ruffman. The children were aged 5 and 6 years and were all healthy and community dwelling within Dunedin. Children were taken to Southern Community laboratories by their caregivers and 2ml of their blood was drawn by a phlebotomist. Blood was collected from Southern Community laboratories band left to clot at room temperature for no longer than 45 minutes from the time of sampling. The clotted blood was separated by centrifugation at 5000 xg (Hermle Z300 centrifuge) and the serum stored in aliquots at -80°C.

In addition to recruiting the children for blood tests, Dr Kirstie Morgan also measured the height and the weight of the children. Height was measured to the nearest 0.1cm using a portable stadiometer (Seca 214) and weight was measured to the nearest 0.1 kg using electronic scales (Seca 770 High Capacity Scale). Caregivers provided additional information including parental heights.
7.1.3 Women cohort

Serum from 33 women (aged 19-39) was obtained from a pre-existing vitamin D study run by Dr Lisa Houghton in which participants took a daily dose of 1000IU of ergocalciferol (vitamin D2), 1000IU of cholecalciferol (vitamin D3) or a placebo (no vitamin D) for six months during autumn and winter. The exclusion criteria for this study was 1) having a BMI greater than 25kg/m² 2) taking a overseas holiday, using sunbeds or taking their own vitamin D supplements within two months of the study. Participants of the original study were contacted via E-mail to seek permission to use their stored serum with the approval of the University of Otago Human Ethics Committee. Participants were not contacted if they were near menopause (older than 40 years) or if their weight changed by more than 4kg during the course of the study. The latter condition was used because of conflicting reports that AMH levels correlates with body mass and vitamin D levels are influenced by fat mass [270, 322-324].

One woman was excluded after analysis because she had a low baseline AMH (less than 3.6 pmol/L) indicating that she may have started the menopause transition. Because the original study did not collect information on the woman’s menstrual cycle, AMH levels were measured at random throughout the menstrual cycle. This is unlikely to affect analysis as AMH has no detectable variation across the different phases of the ovarian cycle [325, 326].

The vitamin D levels for this group were also provided by Dr Lisa Houghton’s group. These were measured by isotope-dilution liquid chromatography –tandem mass spectrometry according to the method of Maunsell et al [327]. This method measured 25-(OH) vitamin D2 and 25(OH) vitamin D3. The sum of the both forms has been reported in this thesis.
7.1.4 Serum Analysis

Hormone levels were measured in duplicate with commercial ELISA kits as per manufacturers’ instructions (kits are summarised in 7.17). Briefly, this generally involved incubating a precise amount of serum in a commercial ELISA plate pre-treated with a primary antibody against the ligand being tested. The plate was manually washed to remove unbound proteins and then incubated in a secondary (biotinylated) antibody and a streptavidin horse radish peroxidase conjugate.

After further washing, the plate was treated with TMB (3,3′,5,5′-Tetramethylbenzidine) to create a colorimetric reaction that was stopped with sulphuric acid after a set time. The amount of colour in the plate was measured with a VICTOR™ X3 Multilabel Plate Reader (PerkinElmer) and the data was fitted to standard curves created by calibrator samples on the plate to determine the concentration of the ligand in each plate well.

Over the course of three years, the commercial AMH ELISA kits available continually changed. Therefore, three different kits have been used to measure AMH levels. The MIS/AMH ELISA (Diagnostic system laboratories, DSL-10-14400) was used for child cohort and the first batch of 40 men. The EIA AMH/MIS (Immunotech, Beckman Coulter, A16507) was used for the remaining 182 men from the AAA and Control cohorts, and the entire women cohort. The AMH levels determined by this kit have been reported to give similar results to the previous DSL kit [328] and this was confirmed in this laboratory by measuring over 100 samples from various sources with both kits. The third AMH kit was the GenII ELISA (Beckman Coulter, A73818), a successor of immunotech kit, was used to measure the varicose vein and peripheral artery disease men cohorts. The manufacturers report that these kits give similar results [329] and this was confirmed by measuring serum from various sources with both kits.

Mrs Nicola Batchelor, Ms Marion Weimar and Prof Ian McLennan gave technical assistance during serum analysis.
Table 7.17. Commercial ELISA kits used in this thesis

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Kit</th>
<th>Company</th>
<th>Catalogue number</th>
<th>Analytical sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>Active® MIS/AMH ELISA</td>
<td>Diagnostic Systems Laboratories</td>
<td>(DSL-10-14400)</td>
<td>0.04pM</td>
</tr>
<tr>
<td></td>
<td>EIA AMH/MIS ELISA</td>
<td>Immunotech (Beckman Coulter)</td>
<td>A16507</td>
<td>1pM</td>
</tr>
<tr>
<td></td>
<td>#GenII ELISA</td>
<td>Beckman Coulter</td>
<td>A73818</td>
<td>0.57pM</td>
</tr>
<tr>
<td>Inhibin B</td>
<td>Inhibin B GenII ELISA</td>
<td>Diagnostic Systems Laboratories</td>
<td>A81303</td>
<td>1pg/ml</td>
</tr>
<tr>
<td>SHBG</td>
<td>SHBG ELISA</td>
<td>IBL international</td>
<td>MX52011</td>
<td>0.2nmol/L</td>
</tr>
<tr>
<td></td>
<td>SHBG ELISA</td>
<td>ALPCO immunoassays</td>
<td>11-SHBHU-E01</td>
<td>0.1nmol/L</td>
</tr>
<tr>
<td>25(OH) Vitamin D</td>
<td>25(OH)Vitamin D direct ELISA kit</td>
<td>Immun Diagnostik</td>
<td>K2109</td>
<td>3.2nmol/l</td>
</tr>
<tr>
<td>Intact PTH</td>
<td>Intact PTH(1-84)EIA</td>
<td>ALPCO immunoassays</td>
<td>21-IPTHU-E01</td>
<td>1.72pg/ml</td>
</tr>
<tr>
<td>T3</td>
<td>T3 (free) EIA</td>
<td>ALPCO immunoassays</td>
<td>25-FT3HU-E01</td>
<td>0.05pg/ml</td>
</tr>
<tr>
<td>T4</td>
<td>*T4(thyroxine) (free) ELISA</td>
<td>ALPCO immunoassays</td>
<td>25-FT4HU-E01</td>
<td>0.05ng/dl</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Parameter Testosterone</td>
<td>R &amp; D Systems</td>
<td>KGE010</td>
<td>0.030ng/ml</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Quantikine Human IGF-I</td>
<td>R &amp; D Systems</td>
<td>DG100</td>
<td>0.026ng/ml</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Quantikine Human IGFBP-3</td>
<td>R &amp; D Systems</td>
<td>DGB300</td>
<td>0.05ng/ml</td>
</tr>
</tbody>
</table>

SHBG = sex hormone binding globulin, PTH = parathyroid hormone, T3 = triiodothyronine, T4 = thyroxine, IGF-1 = insulin-like growth factor 1, IGFBP-3 = IGF binding protein 3. # This AMH kit has been demonstrated not to cross-react with inhibin A.*T4 ELISA has documented cross-reactivity with T3 (4.5%), there is no documented cross-reactants for the other ELISA kits.
7.1.5 Calculations

The following calculations were used to calculate body mass index (BMI) and body surface area in the various cohorts. Body surface area was calculated using the Mosteller formula [330].

\[
BMI = \frac{Mass \ (kg)}{(Height \ (m))^2}
\]

\[
Body \ Surface \ area = \left(\frac{Height \ (cm) \times Weight \ (Kg)}{3600}\right)^{1/2}
\]

7.2 Mouse studies

7.2.1 Mice

Mouse colonies were maintained by Mrs Nicola Batchelor in the MIS Neurobiology Group. Mice were housed in M.I.C.E cages (animal care systems) under a 14 hour white light cycle and feed sterilized food ad lib. Genotypes were determined from ear biopsies with polymerase chain reaction using specific primers. All experiments involving mice were approved by the University of Otago Animal Ethics Committee.

In this thesis two types of mice were used; the LacZ reporter mouse and the AMH⁺/- mouse. The LacZ reporter mouse is generated by crossing male LacZ⁺ mice that carry the LacZ transgene (described here [331]) with female AMHRII⁺/Cre (described here [332]) that are heterozygote for the LacZ activator under the control of the AMH receptor (AMHRII) promoter.

The AMH knockout mice were generated by Behringer et al [58]. Matings between AMH⁺/- males and females were used to generate the full spectrum of genotypes (AMH⁺/+ , AMH⁺/- and AMH⁻/⁻) in one litter.
### 7.2.2 LacZ reporter mouse histology

AMHRII+/Cre LacZ + males were mated to AMHRII+/Cre LacZ - females. AMHRII+/Cre LacZ + and AMHRII+/Cre/Cre LacZ + offspring were used as report mice for AMHRII whereas AMHRII +/+ LacZ + mice served as their controls.

Mice were anesthetised with a lethal overdose of ketamine (225mg/kg, PhoenixPharm) and medetomidine (3mg/kg, Dormitor®, Pfizer). Once deeply unconscious, a ventral incision made to expose the heart and fixative (4% paraformaldehyde in phosphate buffer solution) was pumped (13.3ml/min) into the left ventricle of the heart for three minutes. After perfusion, tissues of interest were removed and submerged in fixative for two hours at 4°C and then incubated in graduated concentrations of sucrose (0.6M to 2.5M, 4°C) for two days before being frozen in frozen section compound (Leica microsystems) and stored at -80°C.

Tissues were sectioned at 20µM in a cryostat (Frigocut 2800, Reichert-Jung) and thaw mounted on to gelatine coated slides. Slides were fixed for ten minutes in a fixative (2% paraformaldehyde,2mmol/L MgCl₂, 0.63 mmol/L EGTA in phosphate buffer solution) at 4°C and then washed twice for ten minutes in a wash buffer (2mmol/L MgCl₂ in phosphate buffer). Tissue was then stained in an Xgal solution (61.2µmol/L Xgal (Bioline), 2mmol/L MgCl₂, 19.4mmol/L Potassium ferricyanide, 22.8mmol/L Potassium ferrocyanide in phosphate buffer solution) at 37°C for 4 to 16 hours. Fetuses were collected from perfused pregnant mice and processed the same as above. A biopsy from the tail of the fetus was removed for genotyping.

After staining slides were cover slipped with a glycerol solution (90% glycerol, 10% 0.01M phosphate buffer 7.2) and photographed on a Zeiss Axioplan microscope (Zeiss) with associated camera and software. Large sections have been reconstructed by photo stitching a number of photographs together as noted in figure legends; the empty background of the photographs was removed by standard cropping in these cases. Images of tissues have not been altered.
Photographic figures were prepared using GNU Image Manipulation Program (GIMP®) 2.6 (Open source, www.gimp.org).

### 7.2.3 Growth study

AMH\(^{+/-}\) male and female mice were paired for mating and allowed to raise at least one litter of pups before being included in the study. Litters in the growth study were culled to four male pups at random on the day of their birth. In nine litters, four male pups were not available so female pups were included to keep the adjusted litter size at four pups. Litters that were naturally smaller than 6 pups were not included in the study. There were twenty litters measured in total.

Mouse pups were weighed on the day of their birth and marked with a subcutaneous injection of tattoo ink to create a tattoo identifier. Pups were left to bond with their parents until day 7 when they were ear-tagged and daily weighing commenced. Weights were measured in the morning on a scale sensitive to 0.01g (Scout Pro SP202, Ohaus Corporation). Most mice were weighed until they were at least 30 days old. However, some mice were culled from the study at 7 and 20 days for a future analysis of their bones. The genotypes of the mice were blinded until after weighing finished. In total there were 20 litters measured with 17 AMH\(^{+/-}\), 31 AMH\(^{+/-}\) and 16 AMH\(^{-/-}\) male mice studied.

### 7.2.4 Bone measurements mice

Bones were collected from mouse specimens stored in the MIS neurobiology laboratory tissue bank. The mice had been perfused with 4% paraformaldehyde and stored in 70% ethanol. Specimens were selected on age and litter size (Table 7.18). Hind legs (right-hand side preferred) were removed with bone scissors by cutting through the femur as close to the pelvis
as possible. Samples were scanned with muscle and skin intact in a high resolution μCT scanner (SkyScan1172, SkyScan, Antwerp, Belgium). Source voltage was set at 55kV with a 0.5 mm Aluminium filter. Sample was rotated 180 degrees with a rotation step of 0.5 degrees. Data was reconstructed in SkyScan “Nrecon” (V1.5.1.4). Bones were measured with OsiriX image viewer (open-source [333]) in multiplanar reconstruction mode. Bones were measured consistently by identifying landmarks in each of the three image windows and aligning the movement axes according to the methods below.

Table 7.18. Characteristics of mice used in bone scan study

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>AMH</th>
<th>n</th>
<th>Age</th>
<th>SEM</th>
<th>Litter size</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>-/-</td>
<td>6</td>
<td>20</td>
<td>0.00</td>
<td>7.17</td>
<td>0.601</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>+/-</td>
<td>6</td>
<td>20</td>
<td>0.00</td>
<td>7.17</td>
<td>0.703</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>-/-</td>
<td>9</td>
<td>137</td>
<td>2.24</td>
<td>7.78</td>
<td>0.494</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>+/-</td>
<td>7</td>
<td>141</td>
<td>2.70</td>
<td>7.29</td>
<td>0.606</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>-/-</td>
<td>7</td>
<td>141</td>
<td>2.52</td>
<td>7.71</td>
<td>0.606</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>+/-</td>
<td>6</td>
<td>140</td>
<td>2.89</td>
<td>6.67</td>
<td>0.211</td>
</tr>
</tbody>
</table>

SEM= standard error of the mean of age or litter size. M=male, F= female. There were no statistically significant differences between groups 1 and 2, 3 and 4, or 5 and 6.

7.2.4.1 Vertebra

The fourth caudal vertebra was identified at the base of the tail by counting from the first caudal vertebrae (which has large distinctive transverse processes). The width was taken mid-length of the vertebra immediately proximal to the distal trabeculae, and was measured between the tips of the transverse processes. The length was taken from the centre of each end. The XY movement axes were aligned on the transverse processes and spinous process, respectively. The
Z axis was centred through the length of the vertebra (Figure 7.45). If the growth plate and epiphyseal bone were visible then the length of the diaphysis bone was measured also. The density line was drawn parallel to the Z-axis line, across the bone wall at the narrowest point ventral to the transverse process (Figure 7.45 Measuring view of the fourth caudal vertebra in OsiriX image viewer).

### 7.2.4.2 Tibia

Tibia width was measured at the distal fibula attachment site, where the fibula first separates from the tibia. The XY axes met at the centre of the tibia with X axis crossing through the centre of the fibula. The width was taken across the Y axis. The Z axis was rotated to give a vertical cross section of the tibia with the maximum view of the intercondylar depression. The length was taken from the top of the anterior condyle to the tip of the anterior malleolus. In younger mice the length of the bone without the epiphysis was also taken (Figure 7.46). A density line was drawn from the centre of the proximal surface of the bone to the centre of the epiheseal line to measure the density of the cancellous bone. The density of the mid shaft compact bone was also measured as shown on Figure 7.46.
7.2.4.3 Metatarsals

The second, third and fourth metatarsals were counted from the medial metatarsal (identified by the position of the fibula). The XY axis was joined on the centre of the bone at the very distal end immediately proximal to the knuckle. The X axis was centred between the two visible projections of the phalanges. The Z axis was aligned through the vertical centre of the bone. The length was taken from the centre of each end. If the epiphysis was visible then the length of the bone without the epiphysis and growth plate was measured as well (Figure 7.47). The density line was drawn through the anterior surface of the medial cuneiform and through the wall of each metatarsal at the Z-axis line (Figure 7.47).
Figure 7.45 Measuring view of the fourth caudal vertebra in OsiriX image viewer. Top left pane = XY window, bottom left Pane = Z window, right pane = Viewing window. Purple = X-axis, Blue = Y-axis, Orange = Z-axis. Green lines are sites of length measurement, pink are density measurements.

Figure 7.46 Measuring view of the tibia in OsiriX image viewer. Top left pane = XY window, bottom left Pane = Z window, right pane = Viewing window. Purple = X-axis, Blue = Y-axis, Orange = Z-axis. Green lines are sites of length measurement, pink are density measurements.
7.2.5 Bone Density

Screenshots were collected from the bone measurements above. Greyscale values were determined in ImageJ (ImageJ 1.43u, Wayne Rasband, NIH USA) using screenshot images taken in OsiriX during bone measurements. In some images, the axis bars intersected the landmark of interest and bone density readings were unable to be determined.

Landmarks were identified as detailed below, a line was drawn between two landmarks and the greyscale values were collected for pixels along the line. A background measurement was taken from a line drawn in the empty space surrounding the leg. The background value was subtracted from the mean greyscale value for the bone to give a “bone density reading” in arbitrary units.

7.2.6 Vitamin D dosing study

Wildtype (AMH+/−, C57Bl6 background) male mice were lightly anesthetised (0.5mg/kg medetomidine, Dormitor®, Pfizer; and 37.5mg/kg ketamine, PhoenixPharm) before being given...
a 20µl oral dose of either calcitriol in sesame oil (2µg/kg, Rocaltrol®, Roche) or sesame oil alone via oral gavage. Mice were given (2.5mg/kg Antisedan®, Pfizer) to reverse the anaesthesia and monitored on a heated surface until awake. One mouse developed a wheeze while unconscious and was euthanized before regaining consciousness. All other mice tolerated the procedure well. Mice were euthanized by cervical dislocation three days later and their testes were dissected. Testes were snap frozen in liquid nitrogen and stored at -80°C until being processed.

Extraction of mRNA from testes was performed using the commercial RNA extraction kit (RNACqueous® kit, Ambion® Invitrogen) as per the manufacturer's instructions. Frozen testes were ground in a mortar and pestle before being transferred into the RNACqueous kit lysis buffer and homogenised with a homogeniser (Ultra-turrax®). RNA was quantified using a Qubit® 2.0 fluorometer (Invitrogen) and quality was assessed by agarose gel electrophoresis. RNA was then treated with DNase (Ambion® Turbo DNA-free™, Invitrogen) and 2ug was reverse transcribed using a commercial kit (SuperScript® VILO™, Invitrogen). Complementary DNA was then quantified again (as above) and the concentration of all samples was normalised. Real time PCR was performed using the TaqMan® probe system (Applied Biosystems).

Predesigned AMH (Mm00431795_g1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915_g1) were used.

AMH expression was normalised to GAPDH (housekeeping gene) expression using the comparative Ct method described in [334].

### 7.3 AMH promoter sequence alignment

*AMH* gene sequences were retrieved from the Nation Centre for Biotechnology Information (NCBI) gene database, the protein coding region of the genes were removed and the non-protein coding regions aligned in the online Clustal W2 multiple sequence alignment tool (www.ebi.ac.uk/Tools/msa/clustalw2) using default settings. At least 300bp of the promoter sequence upstream of the transcription start site was included for each species. Conserved
motifs that were not able to be identified from the literature were given putative annotation using the CISRED database (www.cisred.org).

### 7.4 Statistical analysis

Statistics were calculated using STATA® 11.1 and Graphpad prism. Correlations were compared with parametric and non-parametric analysis and unless stated, results were similar. Where Pearson’s linear correlation is reported $r$ and $p$ values are given. Where Spearman’s rank order correlations have been reported rho and $p$ values are given. If a significant relationship was found its confounders were tested by partial correlation. Student’s t-test results are presented as $p$ values.
Chapter 8: References


